# THE ROLL OF TOLL-LIKE RECEPTOR 4 IN SYNAPTIC INHIBITION IN THE NORMAL AND INJURED BRAIN

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Abstract

Approximately 1.7 million people in the United States sustain traumatic brain injury each year. Up to 30% of patients who have brain injury may also develop post-traumatic epilepsy (PTE). Studies in our lab have shown that brain injury results in cell death and increased neuronal excitability in the hippocampal dentate gyrus, a region important for learning and memory. Additionally, we found upregulation of toll-like receptor 4 (TLR4), an innate immune receptor, that is localized to neurons and specific inhibitory neuron subtypes in the dentate. Activation of TLR4 plays a critical role in inflammatory responses and has been shown to increase risk for seizures by augmenting excitatory AMPA currents after brain injury but has opposing effects in controls. Since TLR4 is expressed on some inhibitory neurons and not others, I hypothesize that TLR4 may also affect overall dentate excitability through modulation of specific inhibitory synapses. Using the Fluid Percussion Injury (FPI) model of concussive brain injury in mice and immunohistochemistry, this study examined the effect of TLR4 signaling on expression of GABAA receptor α1 subunit, a major subtype contributing to inhibitory synaptic transmission in the brain, in the normal and injured brain. We identified trends suggesting that TLR4 signaling could modulate GABA receptor expression in the dentate gyrus. These data trends suggest that TLR4 could regulate the balance between neuronal excitability and inhibition both before and after brain injury. These mechanisms will provide insight into whether TLR4 can be a potential therapeutic target to reduce neurological deficits after brain injury.

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## Introduction

Traumatic brain injury (TBI) results in a dysregulation of cellular processes that contribute to enhanced neuronal excitability in the hippocampal dentate gyrus, upregulation of Toll-like Receptor 4 (TLR4), and epileptogenesis. The dentate gyrus plays a role in learning and memory, but impairments in this region compromise memory function and are linked to epilepsy. The trisynaptic circuit of the hippocampus is mediated by GABAergic inhibition and is responsible for forming the networks that contribute to these factors. Altering $GABA_AR$ subunit expression compromises its ability to maintain overall excitability, thereby facilitating in the generation of seizures. (Lee and Maguire, 2014)

Previous studies have examined the role of TLR4 in modulating non-NMDA glutamatergic currents (Li et al. 2015). These findings suggest that TLR4 signaling is actively involved in increasing dentate excitability following injury. Additionally, contrary to the idea that TLR4 is expressed only in microglia, researchers discovered that TLR4 in the dentate is expressed in neurons and can alter neuronal excitability. (Li et al. 2015) This suggests important roles on hyperexcitability by neuronal TLR4 signaling in the post-traumatic dentate.

The mechanisms that underlie TLR4 signaling can be further elucidated by understanding its role in modulating inhibitory synapses. It has been proposed that activation of inhibitory $GABA_A$ receptors on dentate granule cells is important for filtering inputs from the entorhinal cortex, exemplifying its role in being the "gatekeeper" of the hippocampus (Dengler and Coulter 2016). Inhibition by different types of GABAergic neurons further mediates this precise balance between excitation and inhibition and any dysregulation, specifically in $GABA_AR$ subunit expression, can have adverse outcomes and could increase seizure susceptibility (Lee and Maguire 2014). Therefore, it is important to understand how expression of $GABA_A$ receptors in

the dentate are altered after brain injury and modulated by TLR4 signaling. Specifically, this study examined $GABA_A$ α1, as it is the major synaptic GABA receptor subtype in the hippocampus.

Before delving further into $GABA_A$ expression in the dentate, it is important to understand the anatomy of this region. Figures 1 and 2 shows that the dentate is composed of the hilus, a polymorphic cell layer, the granule cell layer (GCL) containing the somata of the principal cells, and the molecular layer. The molecular layer is where the dendrites of granule cells receive inputs from the entorhinal cortex and is further subdivided into three distinct regions: the inner, middle, and outer molecular layer based on where they receive their inputs from. Additionally, different inhibitory neuronal populations make synapses to granule cell dendrites in distinct layers. Parvalbumin (PV) is an inhibitory interneuron that has axons and axon terminals in the granule cell layer (Hefft and Jonas 2005), as shown in Figure 2. Conversely, Figure 3 illustrates that somatostatin (SST)-expressing interneurons have axons that project from the hilus and terminate in the outer molecular layer. (Freund and Buzsaki, 1996)

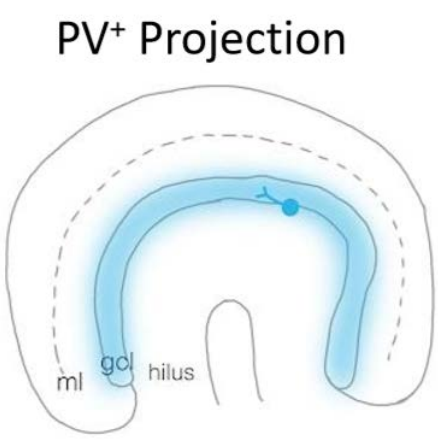


*Figure 1:* Schematic illustrates axonal target of parvalbumin basket cells. Parvalbumin-expressing interneurons have projections within the granule cell layer (GCL).

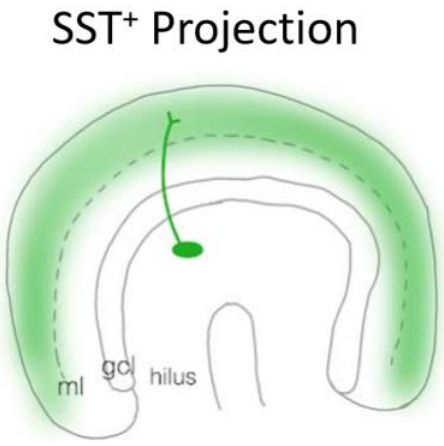


*Figure 2:* Schematic illustrates axonal target of somatostatin interneurons in the dentate. Somatostatin-expressing interneurons make projections from the hilus to the outer molecular layer.

Earlier studies concluded that alteration in synaptic inhibition following brain injury may be caused by selective loss or changes in excitability of various classes of interneurons (Toth et al 1997; Santhakumar 2000). Immunostaining interneurons in the dentate gyrus has helped to demonstrate which inhibitory interneuron co-localizes with TLR4 to understand where this immune receptor is expressed. Pilot data from the lab presented in Figure 4 reveal that there is an overlap between neuronal labeling for SST and TLR4 in both sham mice and injured mice 24 hours after FPI. Conversely, Figure 5 shows no colocalization between TLR4 and PV in sham and injured mice.

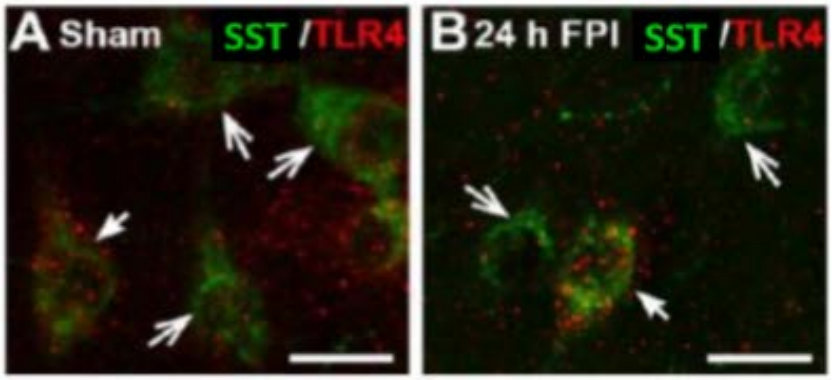


*Figure 3:* TLR4 colocalizes with somatostatin positive neurons: Confocal image of dentate hilar neurons immunostained for somatostatin (SST) and TLR4 (red) shows colocalization in sections from both sham and FPI animals. Scale bar represents 20 µm .

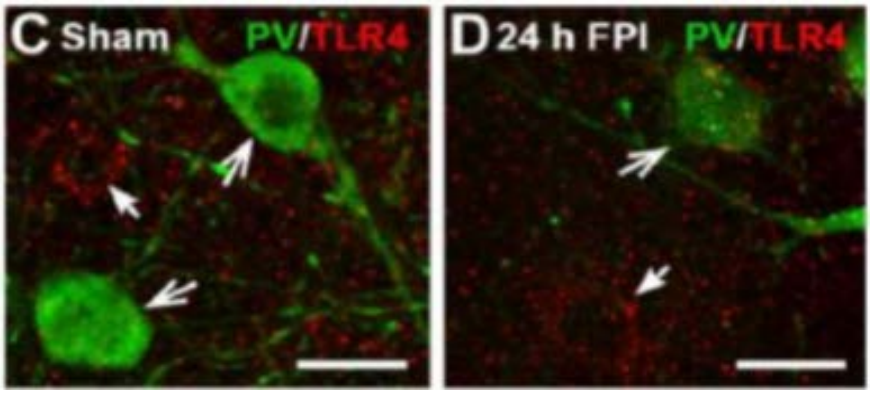


*Figure 4:* TLR4 is absent in parvalbumin positive neurons: Confocal image of dentate hilar neurons immunostained for parvalbumin (PV) and TLR4 (red) shows colocalization in sections from both sham and FPI animals. Scale bar represents 20 µm.

Since we found that TLR4 colocalizes with SST and not PV in the dentate gyrus, we predicted that modulating TLR4 would result in inhibitory synaptic changes in the outer molecular layer, rather than the granule cell layer where PV neurons project. To see these layer-specific changes, our study analyzed alterations in $GABA_A$ receptor α1 subunit expression in the hippocampus following traumatic brain injury (TBI). Understanding this mechanism can help to further identify how modulation of TLR4 can assist in the development of therapeutic drugs to limit epileptogenesis.

## Materials and Methods

*Fluid percussion injury*

Lateral fluid percussion injury (FPI) was performed on mice aged 25-30, following established procedures in the lab. Briefly, mice were anesthetized using isoflourane and placed in a stereotaxic frame to undergo craniectomy. The scalp was incised sagittally and a 2mm diameter hole was drilled into the skull, 1mm lateral and 2mm posterior from bregma and a Luer-Loc hub was attached over the exposed dura. After 24 hours, the mice were anesthetized with isoflourane and attached to the injury device at the syringe hub. Mice underwent a 20msec pendulum-driven pulse at 1.5ATM pressure, indicating a moderate FPI. Sham animals underwent the same craniectomy and were connected to the injury device, but they did not receive a fluid pulse.

*Acute Incubations*

In a first cohort of animals, we conducted acute incubation of live slices from mice one week after FPI or sham injury. Briefly, mice were anesthetized with isoflourane and quickly decapitated, and their brains removed and placed in ice-cold oxygenated sucrose-artificial Cerebral Spinal Fluid (aCSF). 300μm horizontal slices were obtained using a vibratome and transferred to an incubation chamber of 50% sucrose-aCSF and 50% aCSF for 15 minutes at 37°C, then transferred to room temperature to equilibrate for 15 minutes. Slices were then incubated in CLI-095 (10ng/ml) or DMSO control diluted in aCSF for 45 minutes. Slices were continuously oxygenated throughout. After incubation, slices were transferred to a 24-well plate and fixed overnight in 4% paraformaldehyde (PFA) and stored in PBS at 4°C prior to staining. Following immunohistochemistry, we noted that antibody staining failed to penetrate through the entirety of the slice and created a halo when attempting to image using the confocal microscope (Figure 6). Despite multiple attempts to alter immunohistochemical staining and antibody

incubation methods, we were unable to get the antibodies to penetrate through the slice and opted for in- vivo intraperitoneal injection (i.p.) of the TLR4 antagonist to observe changes in TLR4 modulation of $GABA_A$ receptor expression.

*In-vivo intraperitoneal drug injections*

In a second cohort of mice 24 hours after FPI or sham injury, mice were treated with CLI-095 (0.5mg/kg) or vehicle via intraperitoneal injection. Four hours after treatment, animals were perfused with 4% paraformaldehyde (PFA) to allow slices to be prepared for immunohistochemistry. Briefly, mice were terminally anesthetized with Euthasol (1.5mg/kg), and transcardially perfused initially with saline to remove blood and then with 4% PFA to fix tissue. The brain was then removed and placed in 4% PFA at 4°C overnight before storing at 4°C in PBS. 50um horizontal brain sections were prepare using a vibratome and transferred to a 24-well plate containing PBS and stored at 4°C prior to staining.

*Immunohistochemistry*

Following slice preparation, the tissues were stained for $GABA_A$ receptor α1 subunit expression. To start, slices were washed three times for five minutes using phosphate-buffered saline (PBS) and placed on a rocker. After the final wash, slices were incubated for one hour rocking at room temperature in a blocking buffer consisting of 10% goat serum in PBS + 0.3% Triton-X to prevent nonspecific binding.

After 1 hour, the blocking buffer was removed from each well, and the slices were washed three times on the rocker with PBS for five minutes each. Slices were then incubated in the primary antibody to target $GABA_AR$ α1 in 5% goat serum at a dilution of 1:500 (rabbit anti-

$GABA_AR$ α1). The well plate was placed on a rocker in a 4°C refrigerator overnight to allow the antibody to bind. The next day, the slices were washed three times using PBS, followed by incubation with secondary antibody (goat anti-rabbit AF594) at a dilution of 1:1000. The secondary antibody is specific to the animal the first antibody was raised in and is also conjugated to a fluorophore to allow detection of the signal. The slices were incubated rocking at room temperature for an hour before being washed using the same procedure detailed above. The slices were then mounted using a paintbrush and placed on a microscope slide. Once dried, one drop of DAPI mounting medium was added onto each of the slices and a coverslip was placed on top. The slides were placed in the 4°C refrigerator until further analysis.

*Intensity Measurements*

Under a confocal microscope, each slice was analyzed for changes in florescence intensity by using the *Stereo Investigator* software. Raw intensity values were extracted by measuring the entire length of the dentate gyrus from the inner granule cell border to the outside border of the outer molecular layer (OML). Using these values, three calculations for both the infrapyramidal and suprapyramidal sides were obtained in order to measure the average intensity across the entire dentate region. Intensity measurements were averaged separately across the granule cell layer and molecular layer to differentiate between $GABA_AR$ α1 expression from somatically projecting PV+ interneurons and dendritically projecting SST+ interneurons. The layer-specific intensity values were normalized to sham-vehicle levels to allow for comparisons

between FPI-vehicle, FPI-treated with CLI-095, sham-vehicle, and sham-treated mice.

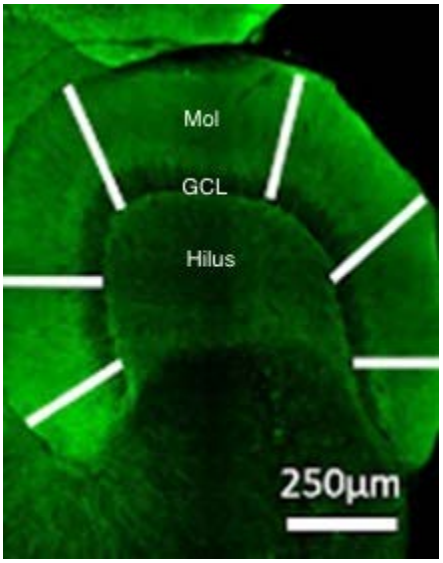


*Figure 5: Measurement of the suprapyramidal and infrapyramidal layers, spanning from the inner border of the granule cell layer to the outside layer of the outer molecular layer.*

*Statistical Analysis*

Data were analyzed using two-way ANOVA (TW-ANOVA) in SPSS. Significance was set to $p < 0.05$. Sample size for intensity measurements is number of animals per group, but due to the low number of animals used, statistical significance was not yet reached. However, we plan to increase sample size to the appropriate number determined by power analysis.

## Results

By understanding neuronal TLR4 expression in the dentate and its role in modulating non-NMDA currents, this study seeks to understand TLR4 modulation of inhibitory synapses. To analyze changes in inhibition, we tested out various antibodies in order to understand how GABAergic neurons were altered. Initially, we tried acute incubation using 300um live hippocampal sections. Given the thickness of the slices, the antibodies did not penetrate well, and we were not able to obtain reliable results (Figure 6).

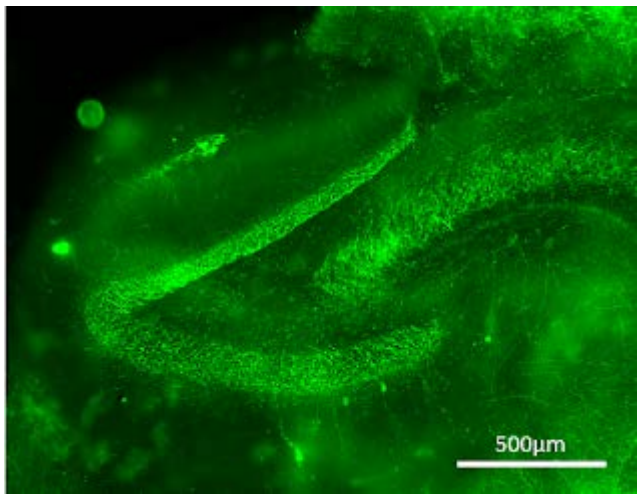


*Figure 6: Confocal image of PV antibody stain in hippocampal dentate gyrus of 300µm slice following acute incubation in TLR4 antagonist showing antibody penetration at top and bottom surfaces only.*

To detect TLR4, we attempted to utilize western blotting to visualize our protein of interest. Several efforts proved to be unsuccessful due to the inability of our gel to transfer to the membrane. After making numerous efforts to understand where the errors occurred, we were still unable to get any noticeable results. In the future, it would be helpful to use this analysis in order to identify the presence of TLR4 in the hippocampus.

Using a separate approach, we found that injecting the mice with TLR4 drugs, CLI-095 and DMSO, following perfusion allowed us to get the desired result. Importantly, reducing the

thickness of the slices to 50µm enabled the antibodies to penetrate the tissue depth, bind to the protein and adequately stain the tissue. After testing various antibodies, our results helped to identify the binding of specific inhibitory interneurons: perisomatically projecting parvalbumin versus dendritically projecting somatostatin. This helped us determine whether TLR4 signaling shows cell-type specific differences in modulating inhibition.

Previous experiments in the lab have demonstrated that, following fluid percussion injury (FPI), TLR4 signaling contributes to an increase in dentate excitability (Li et al., 2015). Since we know that changes in synaptic inhibition following brain injury may be caused by various classes of interneurons (Toth et al., 1997; Santhakumar et al., 2000), we stained for $GABA_AR$ α1 expression in the hippocampus to understand how this expression is altered. By utilizing immunohistochemistry, we were able to see if there were changes in intensity levels across the layers of the dentate. Our data based on two mice per group suggests a trend toward a decrease in expression in the molecular layer, notably the outer molecular layer (OML), the projection region for axons of SST+ neurons which colocalize TLR4 (Figure 7). In contrast, there was no change in GCL where PV axons colocalize.

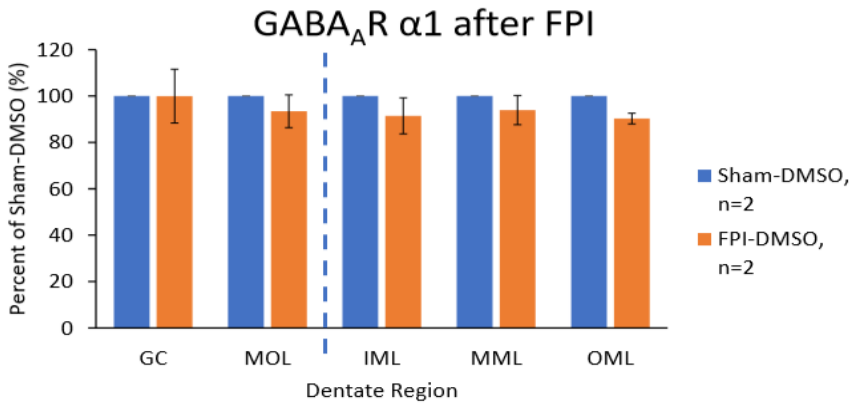


*Figure 7:* **Trend towards a decrease in molecular layer $GABA_AR$ α1 subunit expression after FPI. Summary of intensity measurements at various regions are normalized to corresponding sham data.** Molecular layer (MOL) is subdivided into the inner, middle, and outer molecular layer (IML, MML, OML). Immunostaining shows layer specific changes within the dentate gyrus, indicating a reduction in $GABA_AR$ α1 following FPI in comparison with sham mice. Data are presented as mean±sem.

To further understand the role of $GABA_AR$ in modulating inhibition in the dentate, we used CLI-095 to block TLR4 after injury. Injection of TLR4 antagonists following brain injury help to reduce inflammatory responses and dentate hyperexcitability (Korgaonkar et al., 2020 Annals and BBI). Figure 8 shows that occluding TLR4 after FPI indicates a trend towards an increase in $GABA_AR$ α1 expression in the dentate. These results indicate a reduction in post-traumatic epilepsy (Korgaonkar et al., 2020 Annals of Neurology) and suggest a possible therapeutic target to reduce seizures following brain injury.

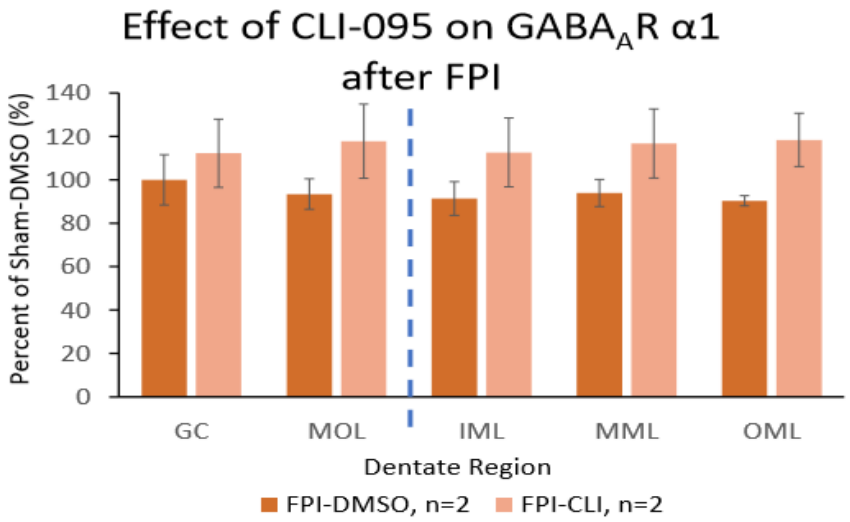


*Figure 8:* **Trend towards increase in molecular layer $GABA_AR$ α1 subunit expression by TLR4 antagonist treatment in FPI mice.** Summary of intensity measurements at various regions are normalized to average FPI data. Blocking TLR4 using CLI-095 after injury increased expression of $GABA_AR$ α1. Data are presented as mean±sem

Conversely, treating sham mice with CLI-095 has been shown to impair memory and enhance network excitability (Korgaonkar et al., 2020 BBI). Therefore, we expected to see a decrease in $GABA_AR$ α1 expression in the dentate. However, our data in Figure 9 shows the opposite effect: there is an increase in expression in sham-CLI mice. Previous studies have

indicated that blocking TLR4 in uninjured mice contributed to cell loss and may be responsible for dysregulating normal brain function (Korgaonkar et al., 2020 Annals of Neurology). Although we would expect to see these outcomes, our preliminary data do not express this trend. However, these experiments were performed using two mice, which shows that we must complete more experiments to increase our n in order to obtain more conclusive results.

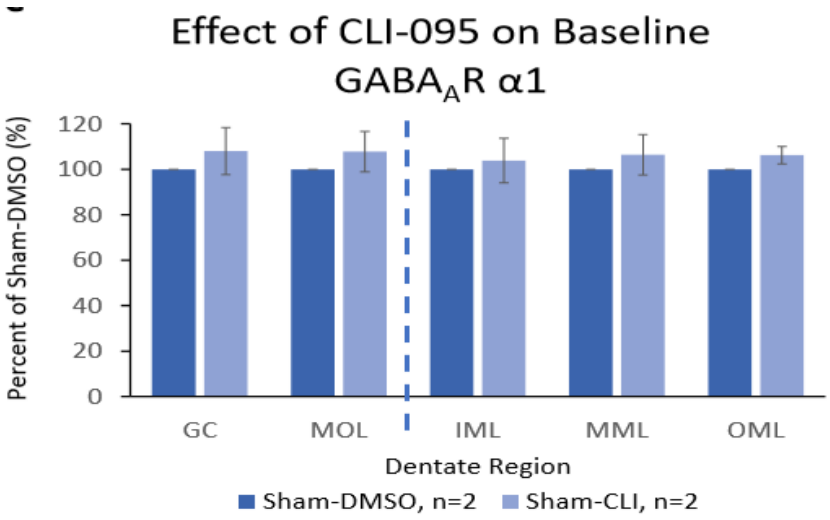


*Figure 9:* **Lack of change in molecular layer GABA$_A$R α1 subunit expression by TLR4 antagonist treatment in sham mice.** Summary of intensity measurements at various regions are normalized to corresponding sham data. Blocking TLR4 using CLI-095 after injury increased expression of GABA$_A$R α1. Data are presented as mean±sem

## Discussion

Prior physiological studies from the lab have identified a role of TLR4 in modulating a balance between excitation and inhibition, which play a part in controlling seizure susceptibility. We have recently found that TLR4 co-localizes with hilar neurons after injury (Li et al., 2015), suggesting that GABAergic neurons express TLR4. In the current study, we find that TLR4 is expressed in a specific class of interneurons: the somatostatin expressing interneurons that project to the distal dendrites of granule cells in the outer molecular layer. In contrast, parvalbumin neurons which project to the granule cell layer do not express TLR4. This study proposed to understand the role of TLR4 in shaping inhibitory regulation of excitability in the dentate in sham and injured mice, by examining expression and layer-specific distribution of specific $GABA_A$ receptors in the dentate gyrus.

Our preliminary data, showing selective reduction in GABA subunits in the molecular layer of the dentate after FPI and the ability of TLR4 antagonist treatment to reverse this trend, is highly promising. It is also notable that TLR4 signaling only altered GABA expression after FPI and not in shams. We must obtain hippocampal slices from more mice for future experiments to confirm these exciting trends. If these trends hold, the current study will have identified a way to target TLR4 to limit epileptogenesis. In line with previous studies in the lab (Korgaonkar et al., 2020 BBI; Korgaonkar et al., 2020 Annals of Neurology), we identified that there is a decrease in molecular layer $GABA_AR$ expression, which could tip the balance between excitation and inhibition following traumatic brain injury. However, blocking TLR4 in injured mice reversed this effect, consistent with the decrease in dentate excitability in earlier studies (Korgaonkar et al., 2020 BBI; Korgaonkar et al., 2020 Annals of Neurology). GABA receptor expression in sham mice treated with CLI-095 to block TLR4 signaling were not different from vehicle treated

controls, which differs from the increase in excitability seen in earlier works (Korgaonkar et. al, 2020 BBI; Korgaonkar et. al, 2020 Annals of Neurology). Additional studies are needed to resolve the mechanisms by which TLR4 alters excitability in sham animals.

Given the physiological change in network excitability in the dentate gyrus, this study used immunohistochemistry to understand how inhibition is also altered. Our data suggested that there is a reduction in inhibition by $GABA_A$ receptor α1 subunit expression. These trends need to be further validated in an expanded study and with functional electrophysiological analysis. Targeting the mechanisms that underlie TLR4 signaling make it an area of interest in order to reduce the onset of seizures in patients who have experienced brain trauma. By examining the role in both excitation and inhibition, our lab demonstrated that TLR4 can prove to be efficacious in reducing epilepsy.

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