UNIVERSITY OF CALIFORNIA SAN DIEGO

Impact of GCaMPx on Pyramidal Neuron Function in Mouse Visual Cortex

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

Nathan DeMarco

Committee in charge:

Professor Brenda L. Bloodgood, Chair
Professor Shelley Halpain
Professor Takaki Komiyama

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ABSTRACT OF THE THESIS

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The mechanisms linking inducible transcription factor (ITF) activity with neuronal connectivity and receptive field properties are of great interest, but poorly understood. Of note, the immediate early gene transcription factor (IEG-TF), neuronal PAS domain 4 (NPAS4), is an ITF that is expressed exclusively in response to neuronal activity or depolarization. In the mouse

visual cortex, NPAS4 is expressed at high levels during the critical period, a time during development in which neural plasticity is increased. To study NPAS4 in the visual cortex, we have elected to use *in vivo* calcium imaging. However, it is not known if GCaMP expression affects neuronal development or the induction of NPAS4. By utilizing *in utero* electroporation, we aimed to introduce GCaMP into the pyramidal neurons of the mouse visual cortex and analyze the function of the cells that were electroporated. We first found that the cells electroporated with GCaMP in early development are neurons. In addition, expression of GCaMP does not impact basal or stimulus-dependent induction of NPAS4 in the visual cortex of mice. Thus, GCaMP can be used to study the impact of NPAS4 expression on neurons *in vivo* without disrupting endogenous NPAS4 induction.

Introduction

Experience-dependent synapse and circuit plasticity is fundamental for learning and memory and underlies behavioral and cognitive flexibility. There are many molecular mechanisms that support plasticity, including engaging activity-dependent transcription factors and gene regulation. However, it is poorly understood how a transcription factor changes neuronal connectivity and how these changes impact receptive field properties *in vivo*. Neuronal PAS domain protein 4 (NPAS4) is an inducible transcription factor (ITF) of the BHLH family and is involved in the reorganization of inhibitory synapses onto pyramidal neurons [3]. One key aspect of NPAS4 that sets it apart from other immediate early genes (IEGs) is that it is expressed solely in response to neuronal activity or depolarization signals, with Ca^{2+} influx as a necessary requirement (Fig. 1). In contrast, other activity-dependent IEGs such as p-CREB or c-fos are activated by a variety of stimuli such as neurotrophic factors, growth factors, or forskolin [14].

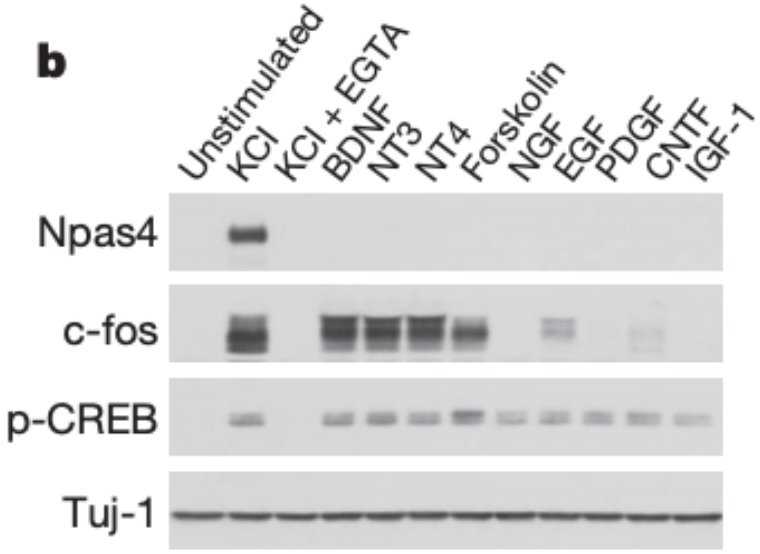

Figure 1. NPAS4 is expressed exclusively by depolarization [14]. Western blot showing that Npas4 is exclusively expressed by depolarization, but not by BDNF, NT3, NT4, forskolin, NGF, EGF, PDGF, CNTF, or IGF-1. Adapted from "Activity-dependent regulation of inhibitory synapse development by Npas4," by Lin Y, Bloodgood BL, Hauser JL, et al. *Nature*. 2008;455(7217):1198-1204. doi:10.1038/nature07319.

Much of our understanding about the mechanisms and functions of NPAS4 arose from studies done within the hippocampus, where the highly organized structure simplifies the analysis. In these experiments, it was shown that NPAS4 coordinates the redistribution of inhibitory synapses made onto a CA1 pyramidal neuron, increasing somatic inhibitory synapses while decreasing inhibitory synapses on the apical dendrites [3,9]. However, the hippocampus receives a broad range of highly processed sensory information that arises from the parietal, temporal, and occipital areas via the entorhinal cortex, as well as the temporal association cortex [1]. Additionally, there is ambiguity in our understanding of how these sensory inputs are transformed into place fields. Thus, it is difficult to determine if or how NPAS4 expression modifies how sensory information is encoded in the hippocampus.

In contrast, the primary visual cortex (V1) receives direct visual information from both eyes via the lateral geniculate nucleus (LGN). In the binocular region of the visual cortex, neurons receive inputs from both eyes. Early in development, the inputs from each eye are broadly tuned to orientation, yet the binocular cells in the cortex must match their orientation tuning through the two eyes in order for the animal to perceive correctly. This time period, which involves a high level of circuit plasticity, is formally known as the critical period. Early studies have shown that after this critical period, an animal is immune to any visual deprivation procedures indicating there is a clear time period in early development in which the neuronal circuits are especially malleable to visual stimuli [10]. With visual experience, and during the critical period for binocular alignment, the inputs from the two eyes are refined such that orientation preferences are matched allowing for the subject to perceive correctly [13,20,21]. This process requires experience, involves local inhibitory rewiring, and occurs during a developmental period with high NPAS4 expression (Fig. 2). However, this process may be interrupted by visual deprivation and is known to be resistant to recovery past the critical period. Despite this fact, recent studies have found that prolonged exposure to an enriched environment past the critical period is capable of rescuing the binocular alignment of orientation preference [13]. Due to its involvement in the regulation of inhibitory

synapses, as well as its induction with exposure to an enriched environment in V1 during the critical period, we propose that NPAS4 may be the driver of this biology.

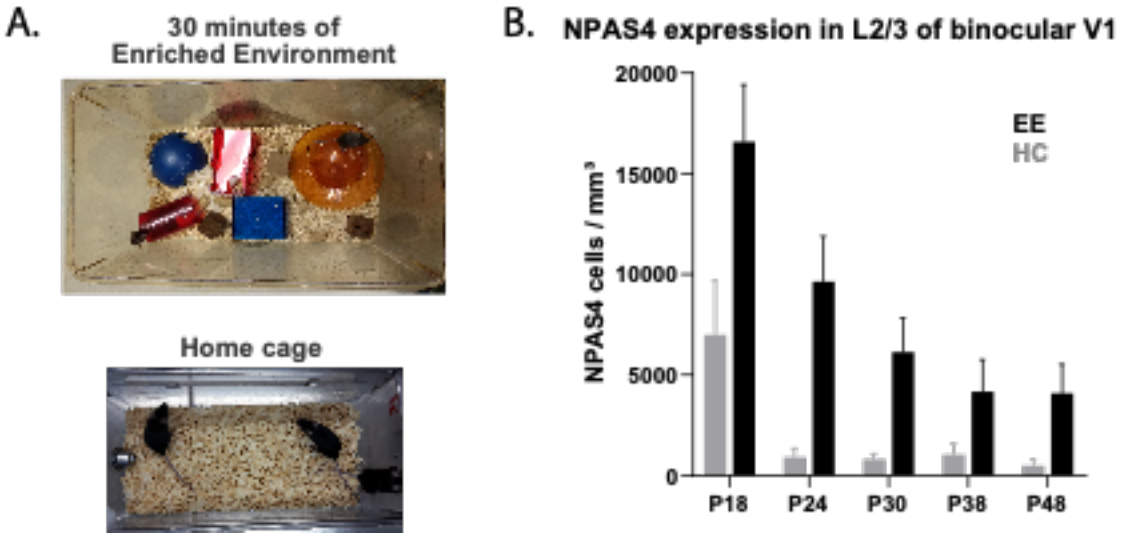

Figure 2. NPAS4 is expressed in response to novel experiences. (A) Mice are moved from a home-cage to an enriched environment to induce expression of NPAS4. (B) NPAS4 expression peaks at P18 in both cages but is markedly higher when exposed to an enriched environment. Adapted from unpublished data from Diego Alvarez in the Bloodgood Lab.

Broadly speaking, the cerebral cortex is organized in six cellular layers that develop at different time-points during gestation. Excitatory neurons are generated in the ventricular zone, as progenitor cells, where they migrate and populate the exterior layers. The oldest neurons are located in the deep portions of the cortex (layer VI) while the youngest are located near the surface (layer II/III) [8]. The middle layer (layer IV) is the primary recipient layer for information from the thalamus and distributes this information to the superficial layers [19]. Due to the sheer interconnectedness of the cortical layers, it is necessary to manipulate the neurons in each layer separately, as manipulation of one layer might affect the others.

As a way to isolate a single layer within the cortex, IUEs have proven to be highly efficient. It is a simple and quick procedure to efficiently perform *in vivo* genetic manipulation of specific cell types at different brain locations [6]. This technique takes advantage of the fact that by targeting neural progenitors at a certain moment during embryonic development, one can address specific populations of newborn neurons that will migrate to a specific cortical layer (Fig. 4). In our experiments the IUE surgeries were performed at embryonic day 15-16 (E15-E16) in order to target layer II/III. On the other hand, region specificity can be achieved by placing the positive electrode of the electroporator over the area to be labeled; in our case the region of V1.

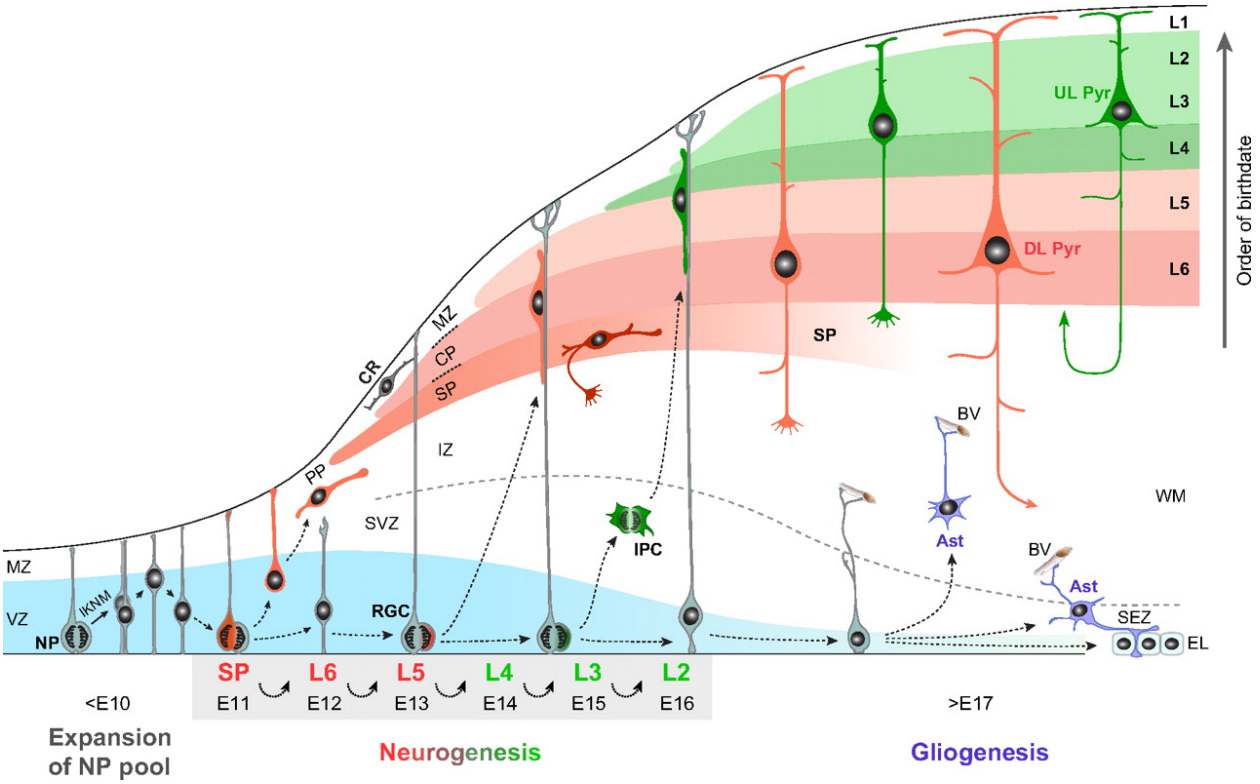

Figure 3. Migration of progenitor cells from the ventricular zone to the cortical layers [12]. Utilizing *in utero* electroporation (IUE), we will be able to target specific layers by using the timeline of development of the cortical layers. To label layer 2/3, IUEs would be performed on E15-E16. Adapted from "Transcriptional co-regulation of neuronal migration and laminar identity in the neocortex," by Kwan, K.Y., Sestan, N., & Anton, E.S. *Development*, 139, 1535-1546. doi: 10.1242/dev.069963.

To visualize the activity, and thus function, of pyramidal neurons within V1, many approaches are available. The number of neurons that can be simultaneously monitored is one of the main factors to consider when choosing the method to visualize cell activity. Among these approaches, *in vivo* calcium imaging stands out as the elected method to simultaneously visualize activity of hundreds of cells in behaving mice for several days [17]. For this specific imaging type, we have proposed the use of the Ca^{2+} indicator, GCaMP. GCaMP is a genetically encoded Ca^{2+} indicator (GECI) consisting of a Ca^{2+}-binding domain and a green fluorescent protein [18]. Since we know that neuronal activity causes the influx of Ca^{2+} into the cytosol via neuronal electrical activity [11], GCaMP is a perfect candidate for showing *in vivo* activity of the neurons as it is activated via this same influx of Ca^{2+} inducing an increase in its fluorescence. However, since GCaMP binds to the intracellular Ca^{2+} used in neuronal signaling, it is not clearly known whether or not this may affect certain downstream signaling, such as NPAS4 expression. More specifically, like all calcium binding proteins, GCaMP acts as a buffer, so this will perturb the same calcium dynamics that NPAS4 induction is dependent on. Additionally, since post-synaptic intracellular Ca^{2+} is integral in many different forms of neuronal plasticity [2], embryonic expression of GCaMP may affect the overall morphological aspects of neurons, such as dendritic branching and dendritic spine density. While GCaMP has been used extensively for *in vivo* calcium imaging and other similar experiments, it is not clearly known whether electroporated GCaMP expression, and its subsequent activity, may have any effects on either the expression of NPAS4 or the morphological development of the pyramidal neurons within V1.

Therefore, the main objective of this work is to examine the basic properties and function of electroporated GCaMP-expressing pyramidal neurons within the region of V1. In the first part, we will examine the capacity of the neurons to express NPAS4 in response to exploration of an enriched environment. In the second part, we will examine the morphology of the GCaMP-expressing neurons and compare it with WT non-GCaMP neurons.

Materials and Methods

Plasmids

For the preparation of the plasmids, an endotoxin-free maxi-prep was utilized to isolate and concentrate the DNA plasmids. Since these plasmids would be injected into the brains of the mice, it was necessary to utilize an endotoxin-free prep to ensure there wouldn't be an inflammatory response while introducing the plasmid. The plasmids were used in a concentration of 2-4 μg/μl. The two plasmids used in this work were CamKII-GCaMP6f (GCaMP version 6f under the control of the CamKII promoter); and Syn-GCaMP7s (GCaMP version 7s under the control of the Synapsin promoter). GCaMP6f and GCaMP7s were chosen based on the fact that they are the two of the latest variations of the GCaMP family. We also elected to utilize the 6f and 7s variations, as they are the highest binding affinity (Kd) and lowest binding affinity, respectively, allowing us to encompass the full range of Kd between the two. Additionally, a CAG-mCherry (red fluorescent protein mCherry under the control of the CAG promoter) was used in combination with each of the GCaMPs as a control plasmid, since its expression allows for recognition that the pups were efficiently electroporated (Fig. 5).

Figure 4. Plasmid maps for CamKII-GCaMP6f (top left), Syn-GCaMP7s (middle), and CAG-mCherry (bottom left) [7,15]. These plasmids were utilized for *in utero* electroporation, where either the GCaMP6f or GCaMP7s plasmid were mixed with the CAG-mCherry plasmid for injection into the embryo plasmid.

In Utero Electroporations

The genetic crosses were established by mating either a female Npas4 conditional knockout mouse (Npas4 cKO/cKO) or a female wild-type mouse with a male Npas4 cKO/cKO mouse. Each subsequent day after placing the females with the males, we checked for vaginal plugs at 7AM to ensure the plugs would still be present from the night before. Once a plug was

found, we separated the female from the male into a separate cage and labeled that time point as embryonic day 1 (E1). Once embryonic day 15-16 (E15-E16) was reached, we prepared the pregnant female for *in utero* electroporation surgery. This time point was chosen on the basis that if the plasmid DNA was introduced to the neural progenitors at that point, they will migrate to layer II/III of the cortex. All equipment that would be handling the embryos was sterilized using a hot glass bead dry sterilizer. Additional cleaning was done by spraying the tools with 70% ethanol. The tools used were ring forceps, surgical scissors, micropipette, hemostat clamp, and surgical sutures. The mouse was placed in a gas chamber containing a high concentration of isoflurane gas. After proper sedation, the mouse was moved from the chamber to the surgery rig, containing a warming pad and its own mouthpiece, funneling additional isoflurane for consistent sedation. Once the mouse was secured to the warming pad, Nair was spread onto the abdomen of the mouse to remove any hair from the surgical area. Additional 70% EtOH was applied to the surgical area to ensure all potential contaminants were eliminated. An incision was made down the midline of the mouse, first on the skin and then along the muscle, making sure to cut along the "línea alba" to prevent excessive bleeding and unnecessary damage to the tissue. The mouse uterine tube was carefully placed outside of the abdomen and onto a damp gauze sheet. To prevent drying out of the organs, warm saline was applied throughout the surgery. Each embryo was injected with the plasmid DNA using a micropipette with a volume of approximately 1-3 microliters. The injection site was on either hemisphere, in the approximate location of the visual cortex. Once every available embryo was injected, they were subsequently electroporated with 5 pulses of a 35-volt current (10 ms each pulse, 1 pulse per second). The negative electrodes were placed on the side of the uterine sac to hold the embryo in place, while the positive electrode was placed on the injection site at the visual cortex. Upon successful electroporation, the uterine tube was carefully placed back into the abdomen of the mouse and warm saline was added to prevent any additional friction. Surgical sutures (Vicryl plus, ethicon suture) were then used to close up the incision. A cocktail of ketamine-xylazine (anesthesia for the recovery of the surgery), fluoxetine

(painkiller), and the antibiotic enrofloxacin (Baytril) was injected intramuscularly into the right leg and arm of the mouse. The incision site was secured further with application of antibiotic ointment and surgical glue. The mouse was carefully placed into a clean cage, with the cage being placed onto a warm mat until the mouse awakened. The mouse was then monitored for the next two mornings after the surgery to ensure there wasn't any damage to the incision site or debilitating pain. Additional painkillers and antibiotics were administered at this time. Upon successful delivery of the mouse pups, at postnatal day 2-3 (P2-3), the pups were examined using a fluorescent scope to determine if the IUE was successful. Any pups that yielded a positive result for fluorescence were placed back with the mother, while the pups that showed no fluorescence were sacrificed.

Enriched Environment

At P18, we moved the electroporated mice from a standard home cage (HC) to an enriched environment (EE) cage containing plastic and wooden toys. The mice were allowed to explore the new cage for 30 minutes to induce a noticeable expression of Npas4 in the pyramidal neurons of V1 [3]. Out of all of the experimental mice, two of them had a homozygous Npas4/Npas4 conditional knockout background. For each condition, GCaMP6f or GCaMP7s, three mice were placed in the enriched environment.

Perfusions

After 90 minutes of the exposure to an EE, the mice were perfused with 20-30 ml of saline solution containing heparin (2500 units/ml) and CaCl (4mM, twice the physiological concentration of Ca^{2+}). Then the tissue was fixed with 30-50 ml of paraformaldehyde 4%. After perfusion the brain was dissected and placed in sucrose 30% as a cryopreservative, until completely soaked in the solution (when the brain sinks).

Microtome slicing

Each brain was sliced at 40 microns and placed in a 12-well plate containing PBS. The slices were further separated by anterior to posterior using another 12-well plate based on the separation of the corpus callosum. Only the slices containing the region V1 were used for the analysis. The slices were then placed in cryopreservative solution and kept at -20 C until it's analysis by immunohistochemistry.

Immunohistochemistry

Slice tissue obtained as previously described was washed 3 times in tris-buffered saline (TBS) for 5 minutes with agitation at room temperature. The tissue was blocked by incubation with TBS++ for 1 hour with agitation. The TBS++ was composed of TBS, 0.25% Triton, and 3% goat serum. The objective of this step is to prevent non-specific binding by blocking the unoccupied spaces of the membrane before introducing the actual antibodies. The tissue was incubated in the primary antibody in TBS++ solution for 48-72 hs. The primary antibodies used were rabb. anti-NPAS4 (1/2000), guinea pig anti-NeuN (1/1000), and chick. anti-GFP (1/1000). The next day, the tissue was washed twice for 15 minutes each in TBS. The tissue was then blocked in TBS++ for an additional 15 minutes. The secondary antibodies used were anti-rabbit Alexa 647 (1/250), anti-guinea pig Alexa 405 (1/250), and anti-chicken Alexa 488 (1/250). The tissue was incubated in the secondary antibody solution for 2-3 hours. After 3 washes with TBS, the tissue was carefully mounted on the slides and mounting media was used to diminish photobleaching and provide stability.

Data Analysis

All images were taken using a Zeiss 880 Airyscan Confocal microscope. 40x water immersion objective with a numerical aperture of 1.2 and a pinhole of 1-1.1 airy units were utilized.

(Credit: University of California, San Diego Microscopy Core). Cell-counting was assessed using FIJI software.

Results

To understand the effects of the genetically encoded calcium indicator, GCaMP, we utilized *in utero* electroporation (IUE) to incorporate plasmids containing GCaMP into the pyramidal neurons of the visual cortex. This requires an extensive process: from finding a vaginal plug, to the point in which the pups are placed in an enriched environment (EE) to ensure induction of Npas4 in the pyramidal neurons of V1 (Fig. 6).

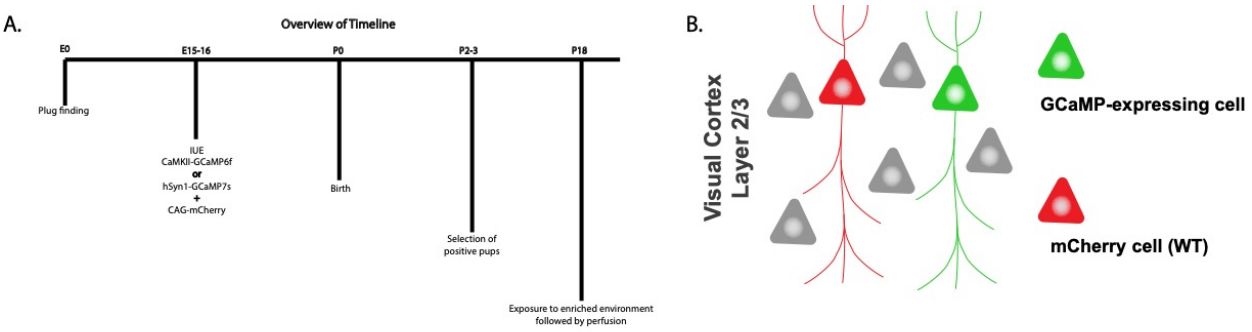

Figure 5. Overview of Experiments. (A) Experimental timeline. The timeline shows *in utero* electroporation performed at embryonic day 15-16 to generate layer 2/3 cells with our plasmid of interest. At post-natal day 2-3, selection of pups positive for mCherry are selected. Finally, at post-natal day 18, the tissue is prepped for various analyses. (B) Visual representation of electroporated cells.

GCaMP cells co-express neuronal marker NeuN.

To start the analysis of the characteristics of GCaMP, we first wanted to know if GCaMP-expressing cells are neurons. Thus, the utilization of NeuN was our elected choice for this specific analysis. NeuN (neuronal nuclei) is a neuron-specific nuclear protein which can be identified by immunoreactivity with a monoclonal antibody, anti-NeuN, and is often used as an indicator for neurons [16]. By utilizing immunohistochemistry techniques, we were able to stain the desired tissue with antibodies labeling both GCaMP, with anti-GFP, and NeuN, with anti-NeuN. This allowed us to deduce which cells are able to co-express GCaMP and NeuN. Using FIJI software,

as seen in Fig. 6 we found that 99.86% ± 0.25% of the GCaMP6f cells also expressed NeuN, while 98.96% ± 0.53% of the GCaMP7s cells also expressed NeuN. This analysis shows that through electroporation, we are targeting neurons and confirms that expression of GCaMP is targeted in neurons.

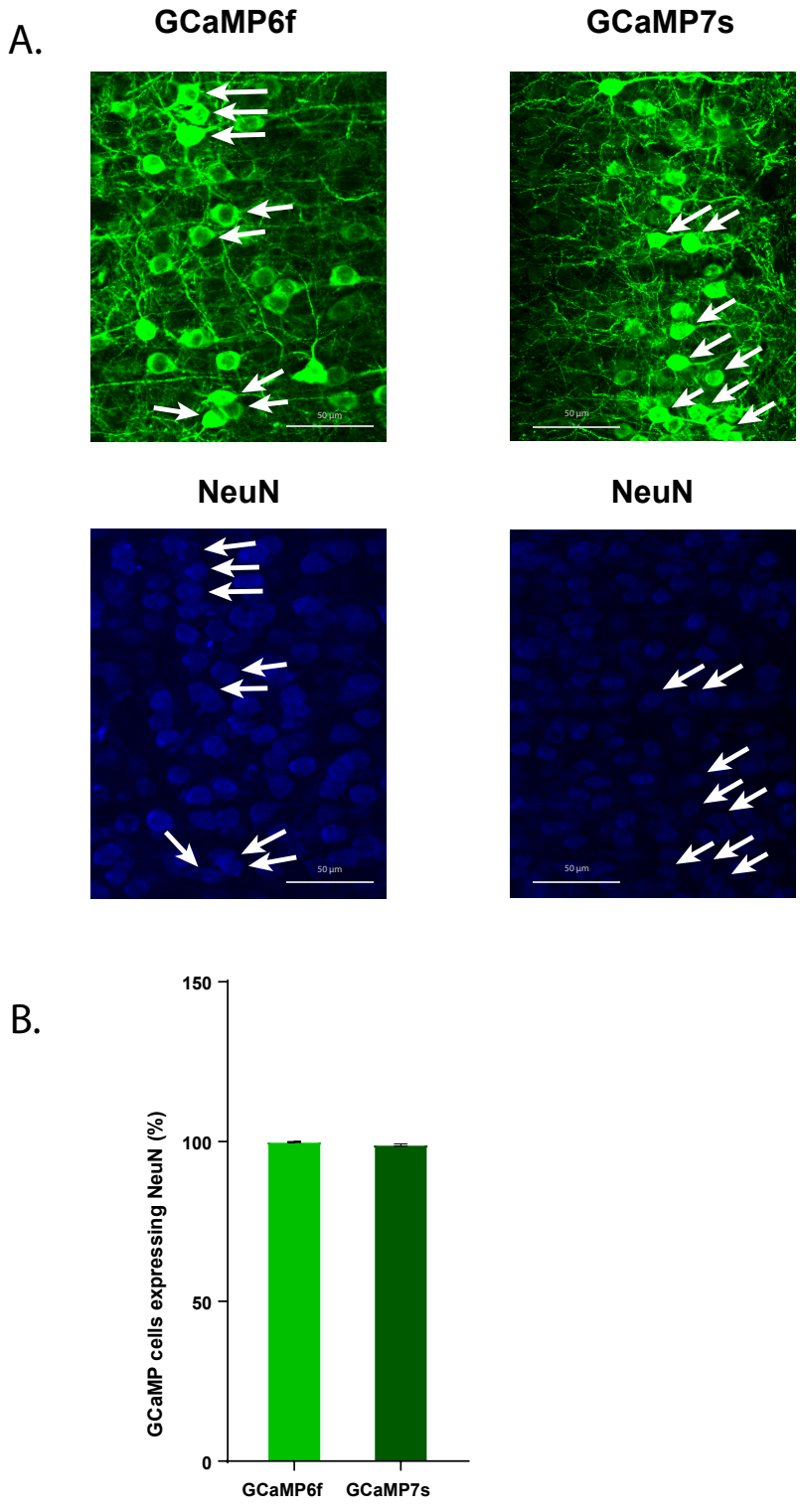

Figure 6. GCaMP cells co-express neuronal marker NeuN. (A) Histology of electroporated cells in mouse V1. White arrows indicate cells co-expressing GCaMP6f/GCaMP7s and NeuN. (B) GCaMP cells were identified and co-expression of NeuN was assessed. 99.86% ± 0.25% of the GCaMP6f cells also expressed NeuN, while 98.96% ± 0.53% of the GCaMP7s cells also expressed NeuN.

The number of NPAS4-positive cells in electroporated mice is similar to non-electroporated WT mice.

To analyze the ability of electroporated brains to express NPAS4, we quantified the total number of NPAS4 cells, in a given volume. For this quantification, we utilized immunohistochemistry by staining the tissue for NPAS4, NeuN, and GCaMP6f/7s. Again, utilizing FIJI, the number of cells that were found to be positive for both NeuN and NPAS4 (WT) were compared with cells positive for either GCaMP6f or GCaMP7s and NPAS4. A Kruscal-Wallis test was performed (p 0.3286) followed by a Dunn's post hoc test. WT vs GCamp6f (p 0.4784) and WT vs GCaMP7s (p 0.3909). As seen in **Fig. 7**, these results show that there is no significant difference between the number of NPAS4-positive cells in electroporated mice and non-electroporated WT mice.

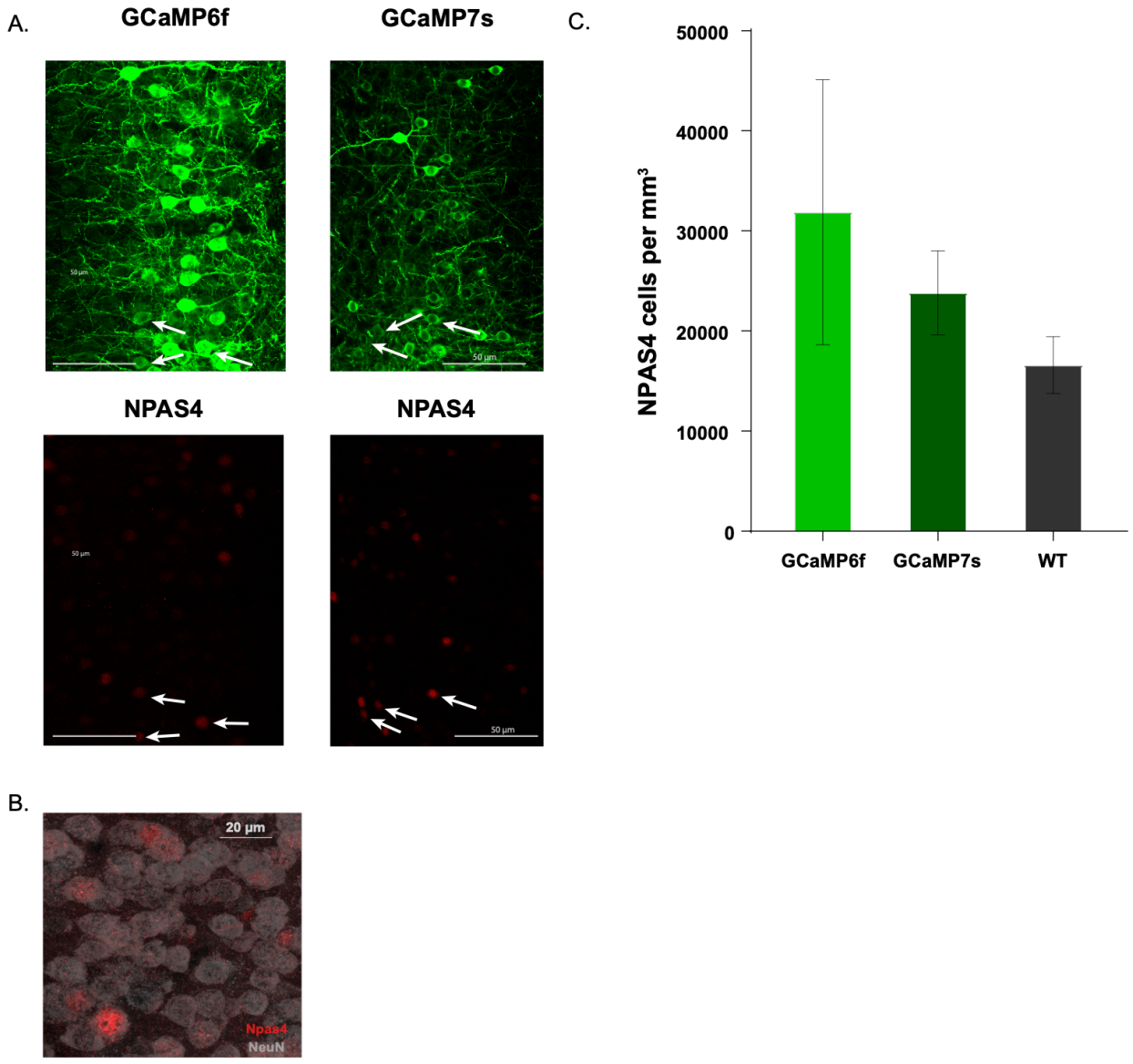

Figure 7. The number of NPAS4 positive cells in electroporated mice is similar to non-electroporated WT mice. (A) Histology of electroporated cells in mouse V1. White arrowheads indicate cells co-expressing GCaMP6f/GCaMP7s and Npas4. (B) Histology of wild-type mouse visual cortex. (C) Mice electroporated with GCaMP6f and GCaMP7s have similar levels of NPAS4 as WT mice. Slices obtained from brains that were electroporated with GCaMP6f and GCaMP7s were stained for NPAS4. A Kruscal-Wallis test (p 0.3286) was performed followed by a Dunn's post hoc test, WT vs GCamp6f (p 0.4784) and WT vs GCaMP7s (p 0.3909).

Cells expressing GCaMP are able to sufficiently express NPAS4.

We next asked if GCaMP-cells can actually express NPAS4. We quantified the number of GCaMP7s cells that were positive for NPAS4 and we compared that with the number of NeuN cells that were positive for NPAS4. We found that 14.95% ± 6.25% of NeuN cells also expressed NPAS4, while 23.9% ± 9.274% of GCaMP7s cells also expressed NPAS4. Wilcoxon test (p 0.25). As seen in Fig. 8, there is no significant difference between the percentage of NeuN cells expressing NPAS4 and the percentage of GCaMP7s cells expressing NPAS4.

Similarly, we quantified the number of GCaMP6f cells that were positive for NPAS4 and we compared that with the number of NeuN cells that were positive for NPAS4. We found that 18.91% ± 9.512% of NeuN cells also expressed NPAS4, while 24.39% ± 7.658% of GCaMP6f cells also expressed NPAS4. Wilcoxon test (p 0.25). From Fig. 9 we see that there is no significant difference between the percentage of NeuN cells expressing NPAS4 and the percentage of GCaMP cells expressing NPAS4.

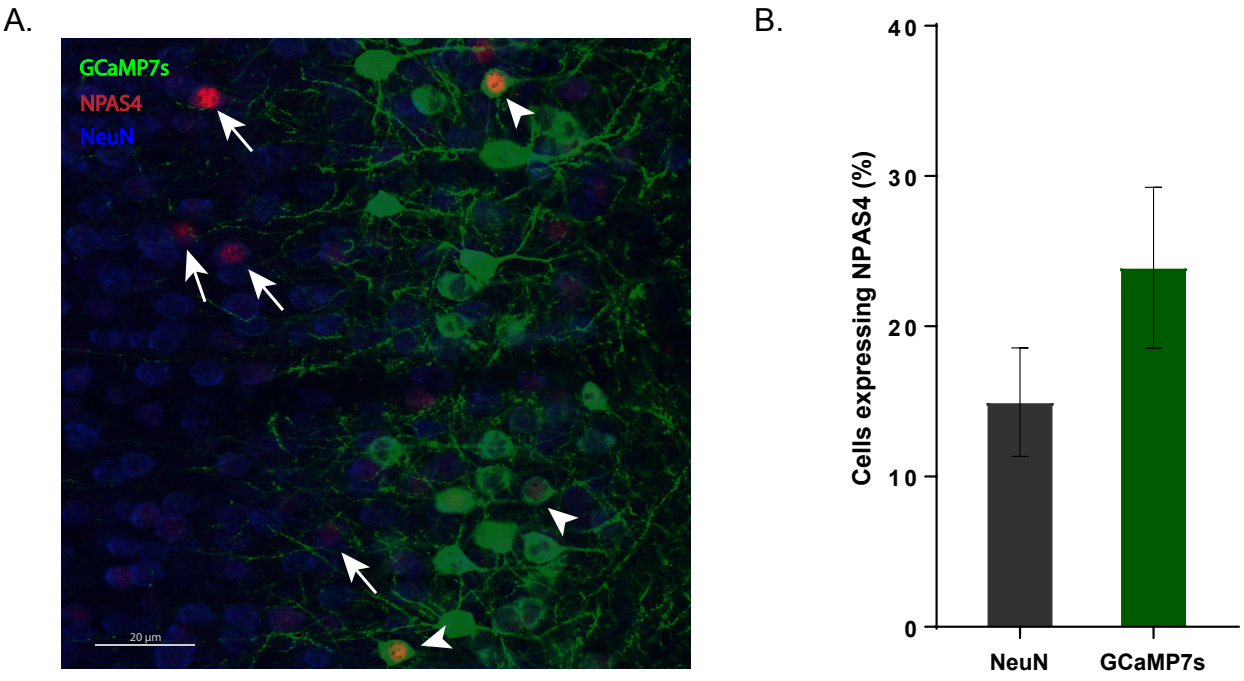

Figure 8. Cells expressing GCaMP7s are able to sufficiently express NPAS4. (A) Histology of mouse V1. Cells positive for NeuN and NPAS4 are marked with arrows, while cells positive for GCaMP7s and NPAS4 are marked with arrowheads. (B) Slices obtained from brains that were electroporated with GCaMP7s were stained for NeuN and NPAS4. 14.95% ± 6.25% of NeuN cells also expressed NPAS4, while 23.9% ± 9.274% of GCaMP7s cells also expressed NPAS4. Wilcoxon test (p 0.25).

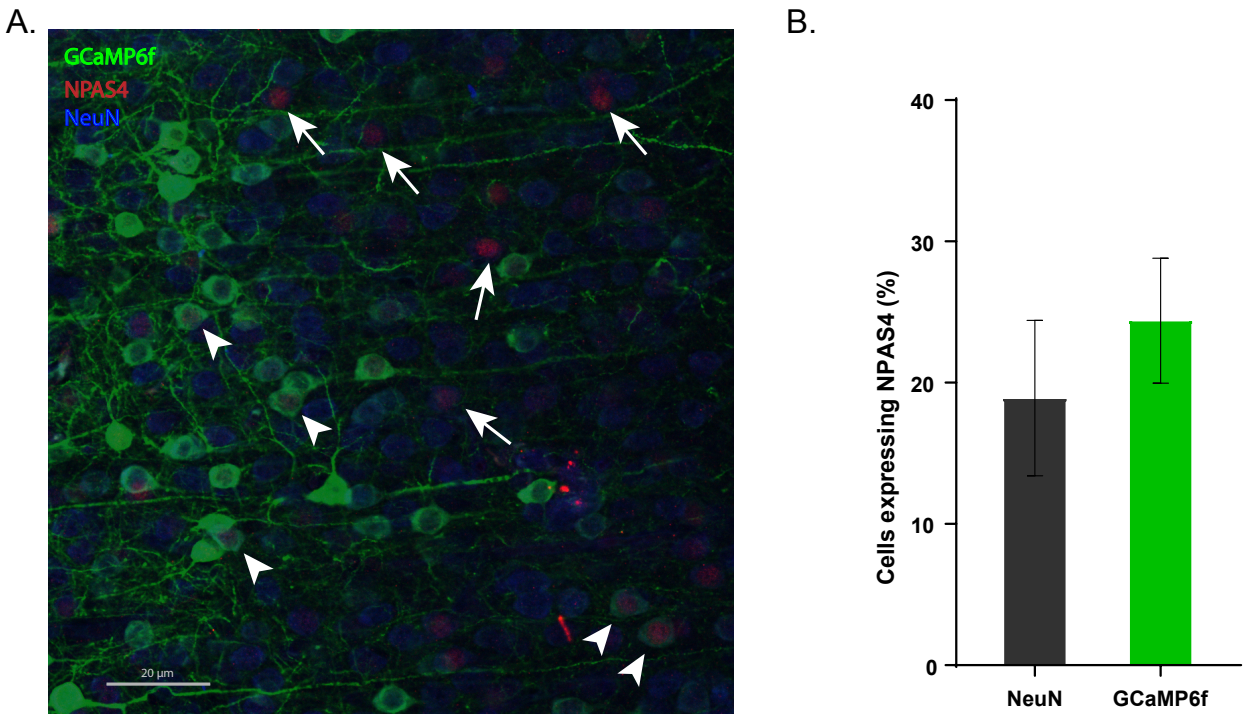

Figure 9. Cells expressing GCaMP6f are able to sufficiently express NPAS4. (A) Histology of mouse V1. Cells positive for NeuN and NPAS4 are marked with arrows, while cells positive for GCaMP6f and NPAS4 are marked with arrowheads. (B) Slices obtained from brains that were electroporated with GCaMP7s were stained for NeuN and NPAS4. Right: 18.91% ± 9.512% of NeuN cells also expressed NPAS4, while 24.39% ± 7.658% of GCaMP7s cells also expressed NPAS4. Wilcoxon test (p 0.25).

Discussion

Although GCaMP has been extensively used previously by utilizing adeno-associated virus (AAV) injections, these experiments were done in postnatal mice [4]. Our experiments aimed to elucidate the effects of GCaMP on the pyramidal neurons of V1 during early development. Utilizing IUEs, and subsequent immunohistochemistry, we found that 99.86% ± 0.25% of the GCaMP6f cells also expressed NeuN, and 98.96% ± 0.53% of the GCaMP7s cells also expressed NeuN. These results highlight the fact that electroporated GCaMP is targeting neurons, as these cells are still able to express NeuN. Therefore, the IUEs are working as anticipated.

After confirming that electroporated GCaMP is specific to neurons, we wanted to confirm that electroporation of the cells in V1 would not affect the ability to express NPAS4. From the results, we see no significant differences in the levels of NPAS4 between cells electroporated with GCaMP6f, cells electroporated with GCaMP7s, and WT cells. This analysis confirms that electroporation does not affect the ability of cells to express NPAS4.

Finally, we wanted to specifically deduce if GCaMP6f- or GCaMP7s-positive cells can sufficiently express NPAS4. From our results, we see there are no significant differences between the percentage of NeuN-positive cells expressing NPAS4 and GCaMP6f- or GCaMP7s-positive cells expressing NPAS4. It is important to note that while there is no true significant difference, we altered the protocol for the perfusion of tissue. After many failed attempts at producing a brain that expresses GCaMP6f or GCaMP7s, we attempted to introduce CaCl (4mM, double the normal concentration) into the paraformaldehyde we were using for the perfusions as a means to boost the signal of any present GCaMP. Upon doing this, we produced several mice that were positive for GCaMP. This may account for the visual, but not statistically significant, differences seen in the figures.

Unfortunately, due to restrictions placed from COVID-19, we were unable to complete any proper experiments to determine if GCaMP would have any effect on the morphology of the

pyramidal neurons in V1. However, we were able to successfully electroporate and perfuse a set of mice that would be viable for this experiment. The analysis is currently on hold. Additionally, we would like to incorporate CLARITY technology, with help from the UCSD SOM Microscopy Core. With this technique, we could utilize intact-tissue imaging of long-range projections along with immunohistochemistry with multiple rounds of staining in non-sectioned tissue [5]. In essence, this would allow us to have a complete reconstruction of a neuron without having to section the tissue completely and risk any damage to the cell we are trying to analyze.

In conclusion, further studies are required to fully understand the role of NPAS4 in the rewiring of neuronal circuitry in the mouse visual cortex during the critical period. There are current experiments in progress which aim to highlight any changes in the electrophysiological characteristics of pyramidal neurons that are electroporated with GCaMP. Despite the analysis that is currently on hold, the results we have found show no significant changes to the expected phenotype of the pyramidal neurons in the mouse visual cortex. This is important as this indicates a potential to utilize GCaMP in future *in vivo* calcium imaging experiments, without any doubts on whether GCaMP is affecting the function of the cells we are studying. If successful, these experiments could reveal the role of NPAS4 in the binocular alignment of orientation preference in V1.

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