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Megha Chiruvella April 15, 2015

The Use of Optogenetics for Cell-type Specific Activation in the Medial Septum

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Abstract

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The synchronized depolarization of hippocampal neurons, known as theta, is present in rats during learning and REM sleep. The MS is the hypothesized pacemaker for the hippocampus and is primarily composed of three types of neurons: cholinergic, glutamatergic, and GABAergic. It is currently unknown which cell type(s) generate theta rhythm. In order to investigate the connectivity between the neurons of the MS and hippocampus, it is necessary to be able to specifically target and activate/inhibit each cell type separately. Optogenetics utilizes viral vectors to specifically express light sensitive ion channels/pumps (LSICs) in particular cell types in the central nervous system, providing millisecond-precision activation or inhibition. Aim 1 of this project is to investigate the specificity of the unique promoter sequences CaMKIIα and hSynapsin as well as the specificity of ChAT-CRE transgenic animals to neuron phenotypes in the MS. Semi-quantitative cell counts can be used to determine the phenotypical breakdown of hSyn-expressing neurons. Aim 2 of this project is to quantify optogenetically activated neurons using the presence of c-fos in the nucleus.

Stereotactic injections of AAV viral vectors were made in the MS, followed by implantation of an optical ferrule targeting the MS. Stimulation procedures utilized LED light. ChAT-CRE-transgenic rats allowed for precise targeting of cholinergic neurons for the expression of either the hChR2 or eNpHR3.0 opsin. The unique promoter sequence CaMKIIα allowed for exclusive targeting of glutamatergic MS neurons for the expression of either opsin. The promoter sequence hSynapin (hSyn) was observed to target all three cell types. When quantified, cells expressing hSyn were identified as GABAergic and glutamatergic significantly more often than cholinergic. Misidentification of GABAergic and glutamatergic MS cells is a concern due to the punctated appearance of both GAD and VGLUT antibody stains; however, it may be that hSyn is expressed in glutamatergic MS cells which project to GABAergic cells within the MS as well.

C-fos was not observed within the stimulated region in any animal but was observed in downstream structures and other regions. This experiment remains inconclusive. Perfusion and fixation quality and stimulation paradigm may have an effect on expression and visualization of c-fos.

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Table of Contents

List of Figures

INTRODUCTION

PART I: The Current State of Epilepsy Therapy and Treatment

Epilepsy is characterized by abnormal amplification and pathologically synchronized neural activity, often manifesting as episodic spontaneous seizures (Bradford, 1995). Early anticonvulsive drugs were typically used for their sedative properties, in order to dampen neuronal hyperactivity (Tower, 1960; Spinks & Waring, 1963). Once the inhibitory function of GABAergic neurons became known, epileptic activity was thought to occur due to shortcomings within the GABA system (Meldrum, 1989; Roberts, 1984), and new drug therapy facilitating GABAergic neurotransmission came about. These new anticonvulsant drugs, such as benzodiazepines, are thought to work because they counteract the localized prevailing excitatory state in the epileptic brain (Bradford, 1995).

Around 30% of all patients with epilepsy are unable to control their seizures even with the use of drugs (Kwan & Brodie, 2000). Patients who have already unsuccessfully been on two antiepileptic drugs have only a 5-10% chance of positively responding to another drug (Kwan & Sperling, 2009). Mesial temporal lobe epilepsy, or complex partial seizures, is the most common type of drug-resistant epilepsy, the standard surgical treatment for which is removal of part of the hippocampus and amygdala (amygdalohippocampectomy) (Drane et al., 2015). In addition to resective surgery, other current therapies for medically intractable epilepsy include vagus nerve stimulation and deep brain stimulation (DBS) of the thalamus; however, the effectiveness of DBS is concerning (Covolan et al., 2014) and resective surgery can produce additional functional side effects, such as cognitive deficits (verbal learning) and memory loss (Helmstaedter et al., 2008; Helmstaedter et al, 2011; Wiebe et al., 2001). The multiple

mechanisms underlying this disease are also poorly known and cannot be understood solely by increasing inhibition via drugs or removing large areas of brain tissue. It is also unknown why anticonvulsive drugs fail in certain cases of epilepsy. For these reasons, there is great need for the development of novel, effective, and function-preserving therapies. We are currently investigating optogenetic methods for a greater understanding of the cell types and connectivity involved in epileptic activity in the tetanus toxin rat model of complex partial epileptic seizures with the future prospect of developing optogenetic epileptic control.

PART II: The Medial Septum is a Target for Hippocampal Control

One type of synchronized depolarization of hippocampal neurons, known as theta, is a ~6 to 8 Hz oscillatory activity usually present in rats and other mammals during exploratory activity or REM sleep (Petsche et al., 1962). Theta rhythm is also associated with synaptic plastic states and is thought to be a favorable condition for encoding information (Vandecasteele et al., 2014). The importance of the medial septum for hippocampal theta rhythm is evidenced by neurophysiological and anatomical connectivity between the two structures (Adey et al., 1958; Andersen et al., 1961; Cragg, 1961; Green and Adey, 1956), the loss of theta rhythm after electrical or drug-induced septal lesions (Green & Arduini, 1954; Mayer & Stumpf, 1958; Stumpf, 1959), and the variable alteration of the hippocampal EEG after high frequency electrical stimulation in the septum (Brücke et al., 1959). The medial septum plays an important role in theta rhythm generation, and is the hypothesized pacemaker for the hippocampus (Hangya et al, 2009). The medial septal neurons may therefore be able to regulate or modulate activity of neurons in the hippocampus. Epileptic seizures are less prone to occur during REM sleep, which is when hippocampal theta rhythm is most present (Montplaisir et al., 1987). It seems, then, that

the presence of theta rhythm creates a functional state during which seizures are inhibited (Colom et al., 2006). Furthermore, generation of theta oscillations has been shown to reduce epileptic activity (Colom et al., 2006) and suppress seizures on demand (Krook-Magnuson et al., 2013)

There are three sub-populations of neurons within the medial septum which project to the hippocampus: glutamatergic (Colom et al., 2005), cholinergic (Lewis et al., 1967), and GABAergic (Köhler et al., 1984) (Figure 1). Excitatory and muscarinic/G-protein-coupled cholinergic neurons and inhibitory GABAergic neurons of the medial septum have long been thought to play an important role in the generation of hippocampal theta rhythm (Bell et al., 2013; Hangya et al., 2009; Nerad & McNaughton, 2006). Early work found that GABAergic neurons of the septum innervate GABAergic hippocampal interneurons and fire in bursts locked to the theta rhythm (~6.5 Hz) (Bland et al., 1999; Brazhnik & Fox, 1997; Green & Arduini, 1954; Stewart & Fox, 1990). Cholinergic septal neurons exhibit slower (~0.3-0.5 Hz), muscarinic or nicotinic-driven rhythmic bursts (Bell et al., 2013; Cole & Nicoll, 1984; Simon et

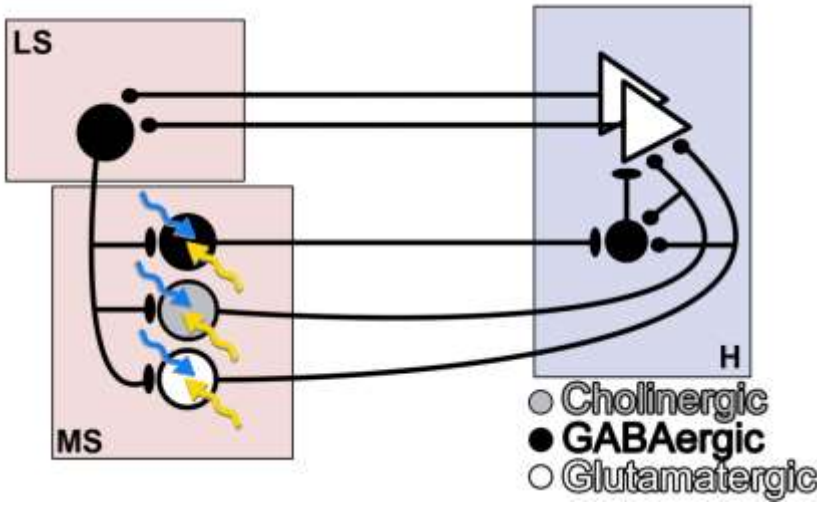


Figure 1 | The septohippocampal network. Schematic representation of the major cholinergic **(gray)**, GABAergic **(black)**, and glutamatergic **(white)** connections in the septohippocampal network between the hippocampus **(H)**, medial septum **(MS)**, and lateral septum **(LS)**. Also represented are targets for optogenetic excitation **(blue arrows)** and inhibition **(yellow arrows)**. Note that this schematic does not fully represent the local connectivity within these nuclei.

al., 2006) which, while not within the typical range of theta rhythm, might still play a major role in generation and control of theta rhythm (discussed further in Part IV). The finding that glutamatergic septal neurons exist and are relevant in the production of hippocampal theta rhythm is more recent (Lin et al., 2003; Simon et al., 2006; Sotty et al., 2003; Wu et al., 2003). Constituting between 4% and 23% of all septohippocampal projections (Colom et al., 2005), glutamatergic septal neurons exhibit slow, slow/cluster, burst, or fast firing properties (Huh et al, 2010; Sotty et al., 2003). Glutamatergic fast-firing neurons tend to spontaneously spike in the theta frequency range (Huh et al., 2010), suggesting that this cell population also provides theta generating input. The connectivity between the cholinergic, GABAergic, and glutamatergic septal populations and hippocampal neuronal activity would benefit from closer investigation to give insight into the workings of the medial septum-hippocampal complex.

PART III: Shining a Light on Optogenetics

Investigating the physiological functionality of neurons entails either activating or inhibiting them to examine their role within the greater neural network. Previously used techniques for neuronal activation include electrical stimulation and pharmacological stimulation. These methods, however, are limited in that the former unselectively affects neurons other than the target population, and the latter has slow kinetics and/or poor reversibility. Optogenetics is a unique technique in that it does not affect surrounding neurons, genetically restricts the susceptibility to stimulation (Zemelman & Miesenböck, 2001), and offers reversible cell type-specific control on a millisecond timescale (Yao et al., 2012). Optogenetics is unlike any existing method of stimulation, and its unique features, in particular, the cell type-specificity,

makes it an excellent tool for selectively targeting and investigating the functional role of the three distinct cell-type populations within the medial septum of the rat brain.

Optogenetics is a novel technique which utilizes viral vectors to specifically express light-sensitive ion channels or pumps in particular types of neurons in the central nervous system, providing millisecond-precision activation or inhibition via LED light (Yizhar et al, 2011) (Figure 2). Neurons have a number of types of membrane channels that are gated by voltage, as well as by temperature and specific ligands, but none that are naturally responsive to light (Banghart et al., 2004). One of the first optical techniques to modulate neuronal activity was to use a light-sensitive "caged compound", whereby a photochemical protecting group renders a necessary ligand, substrate, or second messenger inert, or "caged", prior to photolysis of the protecting group. In the presence of light, the ligand becomes available to activate a neuronal ion channel (Nerbonne, 1996). Modern optogenetics utilizes a less complex method of activating neuronal ion channels. It is known that photosensitive receptors are composed of a protein opsin and an all-trans chromophore retinal (collectively known as rhodopsin) and are found in many organisms, where they are involved in a variety of functions such as animal eyesight, flagellate phototaxis, and invertebrate photophobic responses (Nagel et al., 2003; Ritter et al., 2014). Another early approach to regulate neuronal activity via light was developed by co-expressing *Drosophila* genes encoding signal transduction proteins with a photosensitive rhodopsin, thus resulting in vertebrate neurons with a built-in sensitivity to light (Zemelman et al, 2002). Microbial rhodopsins isolated from the unicellular green algae *Chlamydomonas reinhardtii* – known as Channelrhodopsin-1, a light-gated proton channel (Nagel et al., 2002), and Channelrhopdopsin-2, a light-gated cation-selective channel (Nagel et al., 2003) – have since been genetically expressed in neuronal cells to invoke depolarizing currents in the presence of

blue light within a microsecond timeframe (Boyden et al, 2005). The utility of channelrhodopsin-2 (ChR2), in particular, for optogenetic purposes stems from its fast timeframe (relative to single unit activity) and its innate light transduction ability, with peak activity by light in the 450 to 500 nm range (Yizhar et al., 2011).

Since ChR2 can only elicit action potentials and cannot inhibit natural spiking, this excitatory rhodopsin alone is limiting in its use to examine the functionality and necessity of targeted neurons. It follows that a hyperpolarizing agent with comparable speed and precision would allow for silencing of action potentials in individual neurons. Halorhodopsin, isolated from the archaebacteria *Natronomonas pharaonis* (NpHR), is a light-sensitive chloride ion pump with the ability to induce outward neuronal currents in the presence of light of wavelength 580 nm (Zhang et al., 2007).

Optogenetics is the method of genetically expressing the aforementioned light-sensitive ion channels or pumps (LSICs) in neurons such that the expressing neurons become active or inactive in response to light. A number of viral vehicles can be used to introduce the genetic construct to the neurons of the CNS, such as the adeno-associated virus, recombinant adeno virus, lentivirus, and herpes simplex virus. The choice of which viral vector to use for

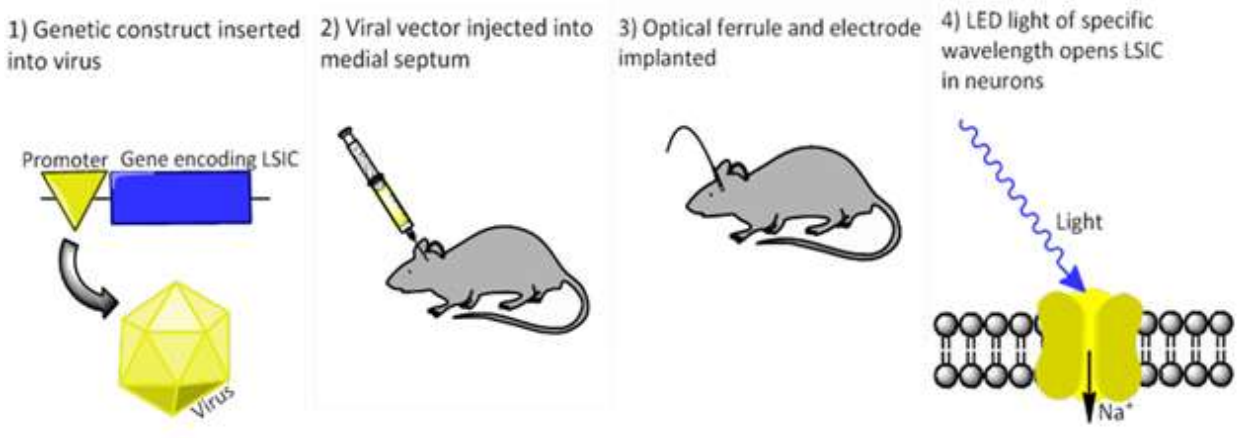


Figure 2 | Overview of Optogenetic Procedure. Genetic construct coupled with a unique promoter sequence is inserted into an adeno-associated viral vehicle (AAV2 or AAV5). Following injection of genetic material into the medial septum (MS) and implantation of LED ferrule, stimulation with LED light produces millisecond-precision activation or inhibition.

optogenetic purposes depends on the characteristics of that vector, including speed, efficiency, and specificity of infection, required titer (concentration of viral particles), and capacity to accommodate the necessary transgenes (Davidson & Breakefield, 2003). Adeno-associated virus (AAV) has been found to rapidly be taken up by specific neurons in the first 30 minutes post infusion, making it a good candidate for optogenetics (Bartlett et al., 1998). AAV is a parvovirus, contains genetic material in the form of single-stranded DNA, and has the capacity to accommodate 4.5 kb of transgenes (Davidson & Breakefield, 2003). There are 12 known serotypes of AAV, with AAV-2 being the most widely used (Govindasamy et al., 2013); however, AAV-5 has been found to exhibit a higher transduction frequency, or distribution, in neuronal cells compared to AAV-2, most likely due to differential tropism (Burger et al., 2004), and it is for these reasons that we use both AAV-2 and AAV-5 vectors.

Cell-type specific expression can be accomplished in different ways: by using a viral vector with selective tropism (Bartlett et al., 1998), by way of unique upstream promoter sequences within the viral vehicle (Kay et al., 2001), or by using custom lines of transgenic animals (Ahmed et al., 2004). A viral vector with a unique promoter sequence preceding the LSIC transgene results in expression of that LSIC only in cell types that have the ability to read that promoter (Davidson and Breakefield, 2003). For example, the promoter sequence CamKIIα is assumed to only be read by glutamatergic neurons; while every neuron within a certain volume uptakes the transgene, only glutamatergic neurons will be able to express the gene, thus selectively targeting a specific cell type.

The nature of Cre-loxP transgenic animals also allows for precise targeting of specific types of neurons via a gene recombination mechanism. Cre is a recombinase that causes recombination of the bacteriophage P1 genome and loxP is a cre-specific recognition and

binding sequence which flanks a sequence of DNA to be excised or inverted by the recombinase (Dymecki & Kim, 2007). By inserting a double-floxed inverted open reading frame (DIO), or reversed opsin sequence, flanked by loxP sites into the genetic construct, only Cre-positive neurons in a transgenic animal will have the ability to invert the promoter and successfully express the gene. Additionally, transgenic animals have been shown to work optimally with optogenetic vectors and techniques (Arenkiel et al., 2007). In these ways, it is possible to selectively express LSICs and activate or inhibit specific cell-types within the brain.

PART IV: Prior Data, Hypothesis, and Aims of this Project

Medial septal cholinergic neurons fire slowly (~0.3-0.5 Hz) compared to theta rhythm (~6-8 Hz) (Simon et al., 2006). However, since these neurons directly influence the hippocampus as well as activate septal glutamatergic neurons, which go on to activate the disinhibitory GABAergic network, cholinergic input may be priming the inhibitory interneurons, keeping them in a subthreshold activation state (Bell et al., 2011; Bell et al., 2013; Wu et al., 2003). Cholinergic input may therefore be providing the underlying background for theta activity to occur, without directly pacing its rhythm. Muscarinic acetylcholine receptor agonists have been observed to induce theta rhythm activity in rat hippocampal tissue sections (Konopacki et al., 1987) as well as in awake animals when delivered via the medial septum (Monmaur & Breton, 1991), while muscarinic receptor antagonists delivered *in vivo* have been shown to hinder learning/memory tasks and block hippocampal theta activity (Givens & Olton, 1994, 1995; Stewart & Fox, 1990). To test the hypothesis that cholinergic septal neurons play a role in modulating hippocampal theta activity, we have previously used optogenetic techniques to selectively activate or inhibit the septal cholinergic population in awake rats. Combining

optogenetics with electrophysiological recordings of the hippocampus *in vivo* allowed for examination of the neural connectivity between this sub-population of septal neurons and the local activity of the hippocampus.

This research performed in our lab indicated that selectively activating cholinergic septal neurons expressing ChR2 with trains of blue LED light (465 nm) of varying frequency and pulse width failed to alter the hippocampal local field potential (net electrophysiological signal). Even taking into account that cholinergic septal neurons are slow-firing and modulating the LED stimulus train to a slower frequency did not demonstrate a change in either hippocampal local field potential. In either scenario, autocorrelation analysis of theta phase showed no changes in theta activity.

It was possible that no response was observed because cholinergic activation was already at a maximum level and any additional optogenetic activation would result in any theta modulations. We then assessed the necessity of cholinergic neurons by optogenetically inhibiting the septal cholinergic population; however, neither rhythmic inhibition by stimulation trains nor constant inhibition was able to demonstrate changes in hippocampal local field potential or theta activity.

Our prior data are intriguing and provide the underlying motivation for this thesis. Specifically, this project investigates two questions, the first of which is: what cell-types are truly being targeted by optogenetic stimulation? In theory, using a specific promoter in the viral vector or using a Cre-transgenic animal would solely activate specific cell-types, but it is important to determine if the opsin-expressing neurons are truly the type we suspect. The CamKIIα promoter (calcium/calmodulin-dependent protein kinase IIα) has been found to be specific to glutamatergic neurons in the hippocampus, but it has never been tested in the medial septum.

Cholinergic neurons contain choline acetyltransferase (ChAT) within the soma, so a ChAT-Cre-transgenic rat line should allow for specificity to cholinergic neurons. The hSyn promoter (human synapsin) has until now been assumed to be nonspecific to cell-type, but recent work has shown that this promoter might actually be specific to GABAergic neurons of the medial septum (Kaifosh et al., 2013). The targeting of GABAergic neurons is of particular interest since excitation of solely inhibitory neurons has been shown to suppress epileptic activity in a mouse model (Krook-Magnuson et al., 2013). Optogenetic specificity of the aforementioned promoter sequences is important to investigate because the use of these promoters affects what cell-types are activated by optogenetic stimulation, and subsequently affect whether the resulting hippocampal LFP and theta rhythm are modulated or not.

The second question this project aims to answer is: are the targeted, opsin-expressing neurons of the medial septum truly being activated (ChR2-expressing) or inhibited (NpHR-expressing) by optogenetic stimulation? To answer this query, the presence of a gene product known as c-fos protein will be examined. C-fos protein is the product of the early-immediate Fos proto-oncogene and is abundant in many types of cells shortly after externally-induced activation (Greenberg & Ziff, 1984; Sheng & Greenberg, 1990). C-fos protein is stored in the cytoplasm of a cell until the cell is activated by growth factors, chemical agents, or neurotransmitter agonists; after cell activation, the protein is translocated to the nucleus, where it dimerizes with the Jun protein to form the AP-1 transcription factor, which binds to DNA to regulate gene expression (Caputto et al., 2014; Curran and Morgan, 1995; Tian and Bishop, 2002; Vriz et al., 1992). The work of Tanos et al (2005) suggests that protein kinases such as p38 are responsible for phosphorylation of the cytoplasmic c-fos protein and ultimately, nuclear translocation of c-fos in HEK-293 cells following UV stimulation.

Antibody staining for the presence of nuclear c-fos shortly after a stimulation session will confirm whether or not neurons are being activated, and if so, how many. The hypothesis for ChR2-expressing neurons is consistent with the findings of a number of recent studies, which have demonstrated an increase in c-fos expression within ChR2 opsin-expressing neurons shortly after optogenetic stimulation (Krook-Magnuson et al, 2013; Jasnow et al., 2013). The literature is limited regarding the use of c-fos in conjunction with the inhibitory opsin halorhodopsin, presumably because c-fos is a positive phenomenon used to visualize cellular activation, not inhibition. C-fos can still be used to confirm activation in disinhibited neurons downstream of inhibited NpHR-expressing neurons. For example, GABAergic MS neurons project to GABAergic interneurons in the hippocampus; if the former are expressing NpHR and are inhibited via optogenetic stimulation, the interneurons should have increased activity and express c-fos. Even within the MS, GABAergic neurons are found to inhibit local neurons of different phenotype; these increased activity of these latter neurons can also be visualized using c-fos.

PART V: Long Term Utility of Optogenetics as a Therapy/Treatment for Epilepsy

Optogenetic experimentation has already provided much insight into the physiological workings of the central nervous system and the pathophysiological workings of diseases such as Parkinsons and epilepsy (Gradinaru et al., 2009; Liu et al., 2012). The work of Tønnesen et al (2009) is one of the earliest studies to show suppression of hyperexcitability and epileptiform activity in *in vitro* hippocampal tissue sections by using optogenetic stimulation via halorhodopsin to hyperpolarize glutamatergic neurons. A number of studies utilizing optogenetics have been done to inhibit many types of epileptic seizures *in vivo* as well (Krook-Magnuson et al., 2013; Paz et al., 2013; Sukhotinsky et al., 2013; Wykes et al., 2012).

Optogenetic approaches have been used "on-demand" to predict and stop seizures shortly before they begin or detect and stop seizures in a closed-loop fashion (Krook-Magnuson et al., 2014). Seizures in a kainate mouse model for unilateral temporal lobe epilepsy were ceased on-demand optogenetically by either inhibiting glutamatergic neurons or exciting GABAergic neurons of the hippocampus after epileptiform activity was detected online with custom-designed software (Krook-Magnuson et al., 2013). From a medical viewpoint, on-demand optogenetics limits any negative side effects associated with treatment, as this system would only be in use during and immediately prior to epileptic seizures.

As these efforts to suppress seizures with optogenetics scale to species with larger nervous systems, such as primates or humans, it is important to note that seizures affect a larger volume of brain tissue. Krook-Magnuson et al (2013) have discussed that suppressing behavioral seizures is more difficult than suppressing electrographic-only seizures (with no complementary behavior) due to the sheer volume of brain tissue that must be inhibited; bilateral stimulation of inhibitory neurons was necessary to take on this goal. It is for this reason that optogenetic approaches should target "bottleneck" brain regions, or regions that project to a large volumetric area in human epileptic patients.

There are a few uncertainties that come with the prospect of using optogenetics for epilepsy therapy in human patients, such as whether expressing opsins in the human brain is safe and stable, how a seizure detection device can be safely implanted in humans, and how the light stimulus would be delivered (Krook-Magnuson et al, 2014). While the clinical implications and benefits of optogenetic techniques are yet to be seen in human patients, optogenetic studies and experiments are a powerful source of knowledge regarding the brain's connectivity, the cell-types involved in seizure induction and suppression, and other general underlying workings of pathological neuronal activity.

METHODS

Experimental and Control Groups

For the neuron identification portion of this project, four (4) hSyn animals, two (2) CamKIIa animals, and one (1) ChAT-CRE animal were immunohistologically stained with cell-type specific antibodies.

For the c-fos portion of this project: (1) expressing LSIC, but not receiving stimulation, (2) not expressing LSIC, but receiving stimulation, and (3) not expressing LSIC and not receiving stimulation. Three (3) hSyn-eNpHR, one (1) hSyn-ChR2, one (1) CamKIIa-eNpHR, and one (1) DIO-ChR2 experimental animals were used.

Surgeries

2-month old adult male Sprague-Dawley rats (250-300g) were purchased from Charles River Laboratories (Wilmington, MA, USA). 2-3 month old adult male Long-Evans rats were bred as the non-expressing cage-mates of a Chat-CRE transgenic rat colony. 2-3 month old (250-300g) adult male ChAT-CRE Long-Evans rats (Witten et al., 2011) were bred from our transgenic rat colony (founder male courtesy of Dr. Karl Deisseroth). All animals were maintained within a 12/12 light/dark cycle vivarium with unlimited access to food and water. This work was conducted in accordance with Emory University's Institute for Animal Care and Use Committee.

Each subject underwent two surgical procedures. The first survival surgery introduced the optogenetic viral vector to the stimulation target – the medial septum. Rats were anesthetized with 1.5-4% inhaled isoflurane, and a craniectomy was made 0.40 mm anterior and 2.00 mm

lateral to bregma on the right side of the skull. A pulled-glass pipette attached to a stereotactically mounted injector (Nanoject; Drummond Scientific Co., Broomall, PA, USA) was used to inject 1.8 μL of 10^{12} particles/mL of one of the following: AAV5-hSynapsin-hChR2(H134R)-EYFP, AAV5-hSyn-eNpHR3.0-EYFP, AAV2-CaMKIIα-eNpHR3.0-EYFP, AAV5-EF1a-DIO-hChR2(H134R)-mCherry, AAV5-EF1a-DIO-eNpHR3.0-EYFP (UNC Vector Core Services, Chapel Hill, NC, USA), or AAV2-CaMKIIα-hChR2(H134R)-EYFP (courtesy of Dr. Michael Kaplitt). The injection was made at a 20° angle to the dorsal-ventral axis (0.40 mm anterior, 2.12 mm lateral at the 20° angle, 5.80 mm ventral to pia along the rotated axis) in order to target the medial septum without damaging the medially-located central sinus. After 5 minutes of equilibration the injection was made over 7 minutes with the pipette remaining in place an additional 10 minutes post-injection to prevent reflux. Once withdrawn, the scalp was stapled closed, ketofen was administered as an analgesic (3-5 mg/kg) to minimize pain, and the rats were quarantined for 72 hours before returning to normal housing.

The second survival surgery was performed two weeks later, which we have found to provide ample time for robust channel expression. For medial septal stimulation, a second craniectomy was made over the right dorsal hippocampus centered at 3.50 mm posterior and 2.80 mm lateral to bregma. The dura was incised with a sterile curved scalpel blade. The micro-electrode (MEA) array was positioned at a 50° angle to midline, with the posterior end swung laterally, to match the positioning of the hippocampal pyramidal cell layers (Rolston et al., 2010). The MEA was lowered while simultaneously recording single unit and local field potential activity to attain the ideal positioning (Rolston et al., 2009). When the electrophysiologic recordings stabilized, the original injection craniectomy was reopened, and a calibrated optical fiber ferrule was implanted at a 20° angle to the dorsal-ventral axis (0.40 mm

anterior, 2.12 mm lateral in the rotated axis). Stimulation was performed as the ferrule was implanted, with the resulting recordings immediately analyzed spectrographically. Descent was halted when the optical ferrule reached a depth of 5.50 mm from pia along the rotated axis.

Optical Stimulation

The Gross Lab developed an open-source hardware and software platform, NeuroRighter, for conducting open and closed-loop neural stimulation experiments. Stimulation sessions took place in a 2.5 ft x 2 ft open enclosure filled with fresh bedding to encourage exploratory behavior. To stimulate awake and behaving animals, calibrated ferrules were connected via armored patch fiber cables (200 μm diameter, 0.67 NA, Plexon). NeuroRighter enables custom-designed stimulation times and amplitudes to be defined via Matlab script (Laxpati et al., 2013; Newman et al., 2013). All animals were connected to the NeuroRighter recording system prior to undergoing stimulation. Channelrhodopsin animals were stimulated with 465 nm blue light (Plexon, Inc.) in 30 minute epochs of intensity 50I (mW/mm^2), frequency 35Hz, and pulse width 10ms, repeated three times for a total of 90 minutes of stimulation. Halorhodopsin animals were stimulated with 620 nm orange light in 30 minute epochs of intensity 90I (mW/mm^2), frequency 35Hz, and pulse width 10ms, repeated three times for a total of 90 minutes of stimulation. Animals were sacrificed immediately following the 90-minute stimulation session.

Histology

Histology was performed after experimentation to verify light-sensitive ion channel (LSIC) expression. Rats were deeply anesthetized with an overdose of Euthasol (5ml/kg, Virbac, Fort Worth, TX, U.S.A.) injected intraperitoneally. They were then transcardially perfused with

0.9% saline followed by 4% paraformaldehyde in 0.1M phosphate buffer at pH 7.2. The heads, still containing the optical ferrules, were then separated and post-fixed at 4°C overnight. The next day, the brains were dissected out, removed, and cryoprotected with 30% sucrose at 4°C. Frozen transverse (horizontal) sections were made of 50 μm thickness on either a sliding microtome or cryostat and collected in 0.1M PBS.

Sections containing the medial septum were stained for one of the following: c-fos (rabbit anti-c-fos antibody (4): sc-52, Santa Cruz Biotechnology), GAD67 (mouse anti-glutamate decarboxylase 67kDa isoform antibody, Millipore), VGLUT2 (mouse anti-vesicular glutamate transporter 2 antibody), or ChAT (goat anti-choline acetyltransferase antibody). Detailed staining procedure can be found in the supplemental information section. Sections were mounted on glass slides and mounted with Vectashield mounting medium with DAPI (Burlingame, CA, U.S.A.) for visualization of nuclei. All sections were imaged in the NIS-Elements software (Nikon Instruments, Inc., Melville, NY, USA) using a Nikon DS-Fil color digital camera on a Nikon E400 microscope equipped with TRITC, FITC, and DAPI fluorescence cubes.

Identification of hSyn LSIC-Expressing Cells

In order to estimate the number of hSyn LSIC-expressing neurons which identify as GABAergic, anatomically-matched cell counts were used. Two blinded assistants were used for tissue imaging and cell counting. Four 40x images were taken in the medial septum and one 40x image was taken in the striatum, in a GAD67 stained section of each hSyn animal. Four 40x images were taken in the medial septum of VGLUT2 and ChAT stained sections of the same animals. Images taken in the medial septum were not randomized due to the scarcity of hSyn LSIC-expressing cells throughout the entire septum. Both blinded assistants individually

examined the images, and for each, defined the number of hSyn LSIC-expressing neurons, GABAergic/glutamatergic/cholinergic neurons, and colocalized neurons. The imaging program ImageJ was used for cell counts. At times, extremely bright EFYP fluorescence has the potential to bleed into the red (TRITC) channel on the microscope, resulting in the appearance of red and green co-expression. Ambiguous neurons with this bleed through appearance were not quantified.

Anatomically-Matched Cell Counting for C-fos Positive Cells

In order to estimate the number of LSIC expressing neurons, c-fos positive neurons, and colocalized neurons within the entire medial septum, anatomically-matched cell counts were used. Two blinded assistants were used for tissue imaging and cell counting. The midline of the medial

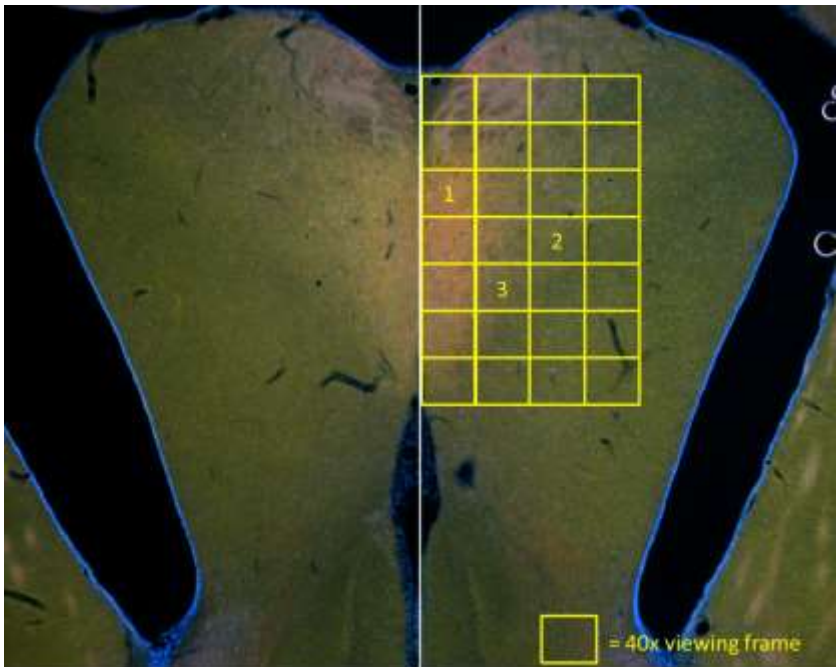


Figure 3 | Anatomically-matched cell counting scheme within the medial septum.
4x image of the medial septum with DAPI (blue), LSIC expression (red), c-fos (green). Midline (white line) and theorized grid of 40z viewing frames (yellow) with arbitrarily pre-selected frames for cell-counting.

septum area for each tissue section was determined (figure 3). To the right of the midline, a grid composed of 40x viewing frames was theorized (figure 3), beginning at the top of the septum, where LSIC expression begins. Three viewing frames within this grid were preselected and defined (figure 3). These three specific viewing frames were imaged at 40x in every section of every animal. Both blinded assistants individually examined the images, and for each, defined the number of LSIC expressing neurons, c-fos positive neurons, and colocalized neurons.

Technical Difficulties

Some technical difficulties, such as risks during surgery and the difficulty of the procedure were alleviated because of the experience of and training provided by graduate students Nealen Laxpati and Jack Tung.

RESULTS

PART I: Histological Verification of Channel Expression, Cell Identification, and hSyn Specificity to Cell Type

Injection of AAV5-hSyn and AAV2-CaMKIIα based viral vectors produced robust expression in the medial septum (Figure 4A/B, and C/D, respectively). Horizontal sections allow for visualization of the axonal projections from the medial septum to the hippocampus. Injection of AAV5-EF1a-DIO viruses into positively genotyped ChAT-CRE transgenic rats produced expression in cholinergic neurons of the medial septum, visualized in coronal sections (Figure 4E and F).

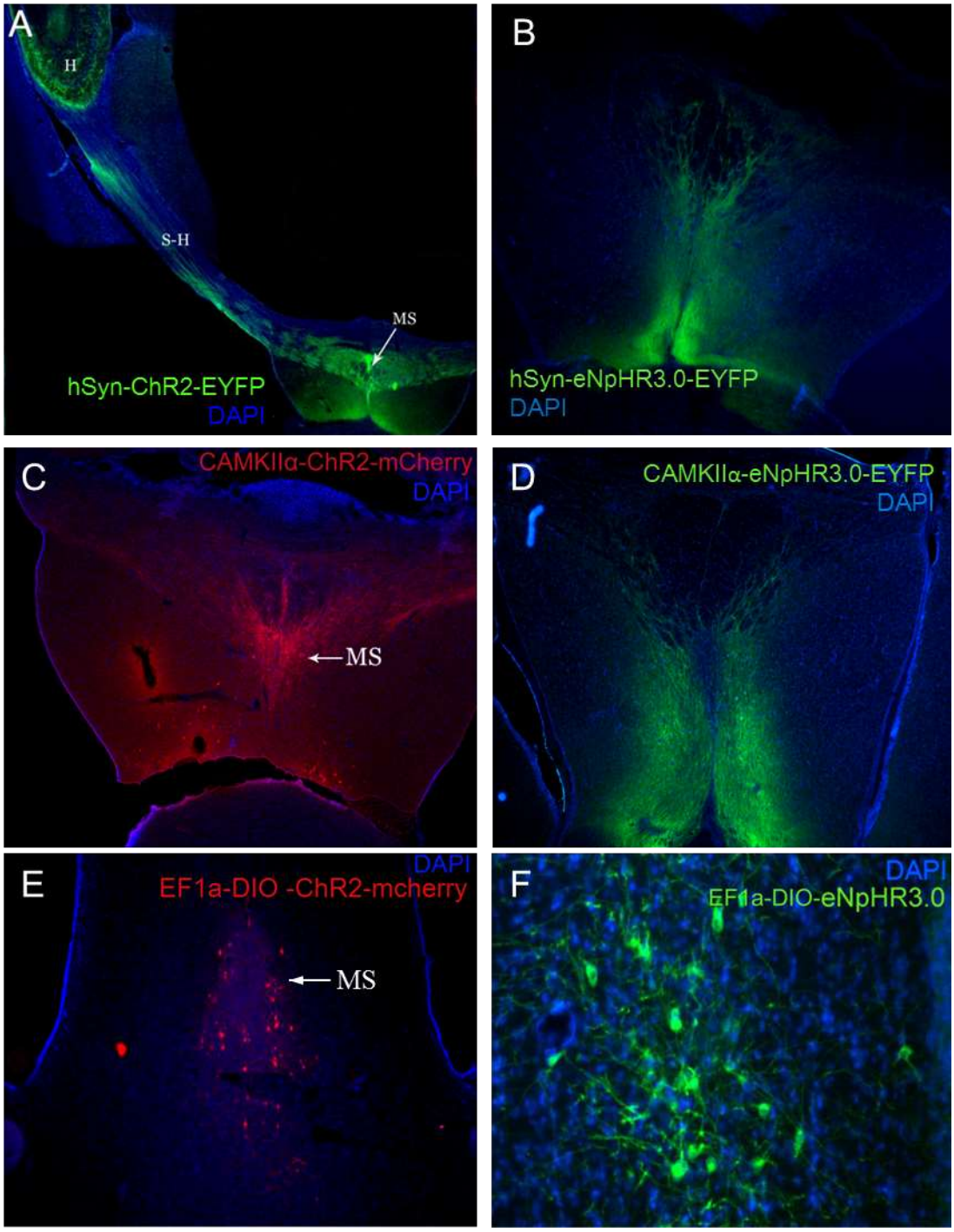


Figure 4 | Robust ChR2 and eNpHR3.0 Expression in the Medial Septum
After injection into the medial septum (MS), hSyn-ChR2-EYFP **(A)** expresses robustly, and projects down the septohippocampal axis (S-H) to the hippocampus (H). Similarly, hSyn-eNpHR3.0-EYFP **(B)** CaMKIIα-ChR2-mCherry **(C)**, and CaMKIIα-eNpHR3.0-EYFP **(D)** produced robust expression in the MS. Transgenic ChAT-CRE rats also express ChR2-mcherry **(E)** and eNpHR3.0 **(F)** in the MS. DAPI, a nuclear stain, is seen in blue.

ChR2-mCherry expression was found to colocalize with identified septal cholinergic neurons labeled with ChAT antibody, confirming cell-type specificity (Figure 5).

Immunofluorescence labeling was performed to confirm selective expression of CaMKIIα-ChR2 within septal glutamatergic neurons. Antibodies against choline acetyltransferase (ChAT, Figure 6A), vesicular glutamate transporter 1 (VGLUT1, Figure 6B), and glutamic acid decarboxylase 67 (GAD67, Figure 6C) were used. Coexpression of CaMKIIα-ChR2-mcherry puncta was observed with VGLUT1 glutamatergic neurons, but was not observed with identified cholinergic and GABAergic neurons.

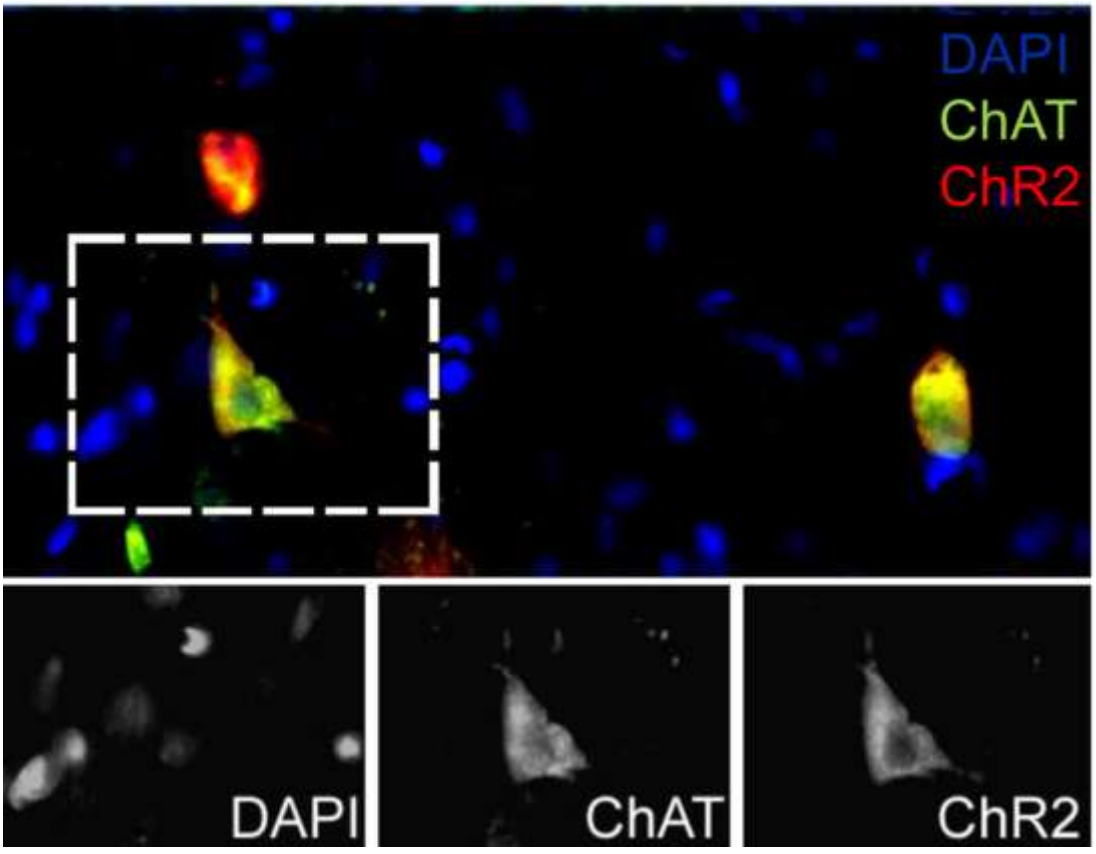


Figure 5| Selective expression of ChR2-mCherry in transgenic Chat-CRE medial septal cholinergic neurons.
A| Expression in the medial septum of AAV5-EF1a-DIO -ChR2-mcherry in ChAT-CRE transgenic rats was readily observed (red) **B|** Medial septal ChR2 expressing neurons (red) colocalized with immunofluorescently labelled cholinergic neurons (ChAT, green). DAPI nuclei stain in blue.

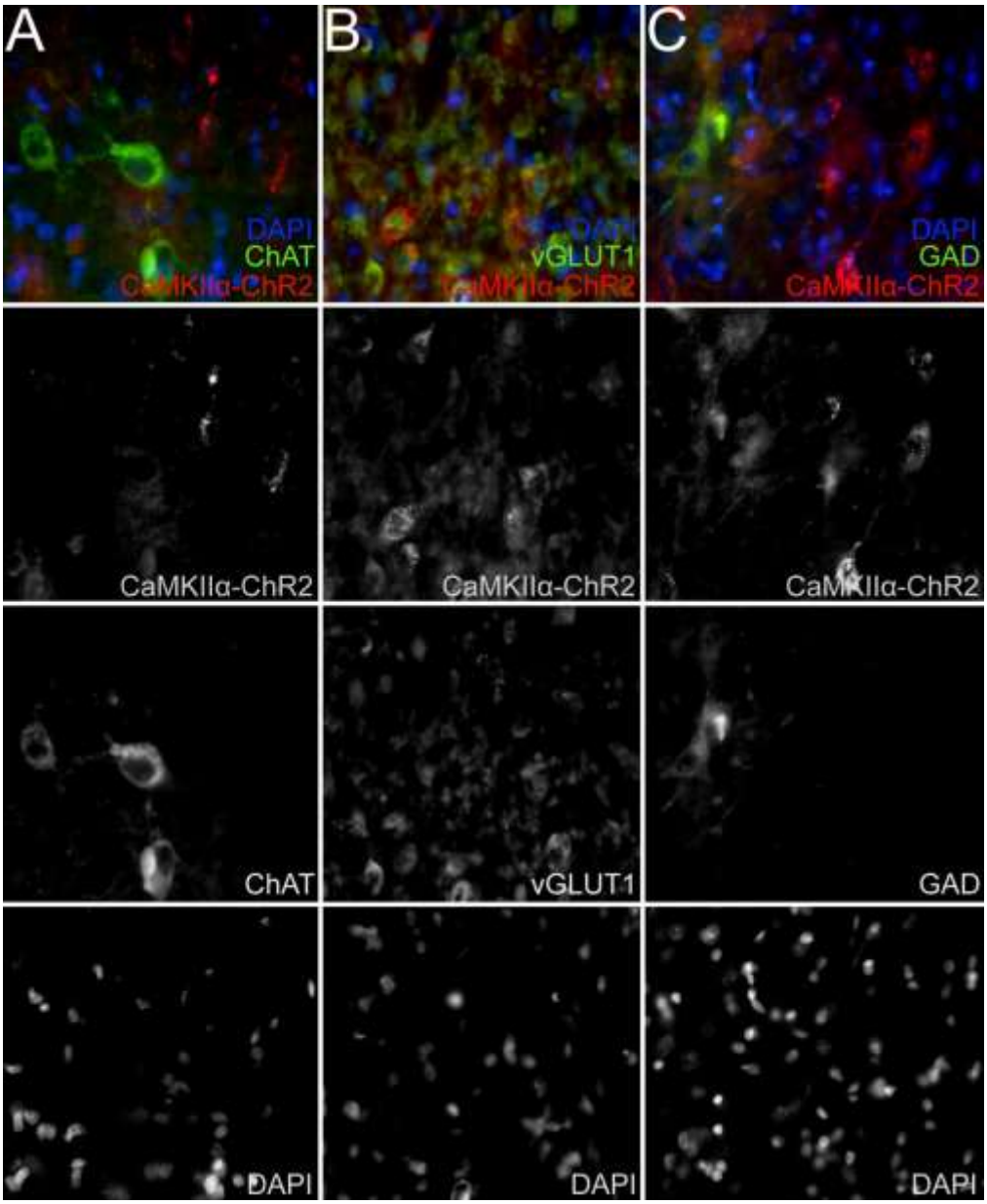


Figure 6 | CaMKIIα-ChR2 selectively expressed in glutamatergic neurons of the medial septum.
CaMKIIα-ChR2 expressing neurons (red puncta) failed to colocalize with immunolabelled ChAT (**A**, green) or GAD (**C**, green) neurons. However, substantial colocalization was seen with the immunolabelled vGLUT1 neurons (**B**, green), suggesting ChR2 was selectively expressed in the glutamatergic cell population in the medial septum. DAPI nuclei stain in blue.

Immunofluorescence labeling was performed to investigate expression of hSyn-eNpHR within septal GABAergic (Figure 7A), cholinergic (Figure 7B), and glutamatergic (Figure 7C) neurons. Coexpression of GAD67 and VGLUT2 immunofluorescent puncta was observed with hSyn-eNpHR expressing neurons (Figure 7A and C, respectively). Coexpression of ChAT antibody was observed with some hSyn-eNpHR expressing neurons (Figure 7B).

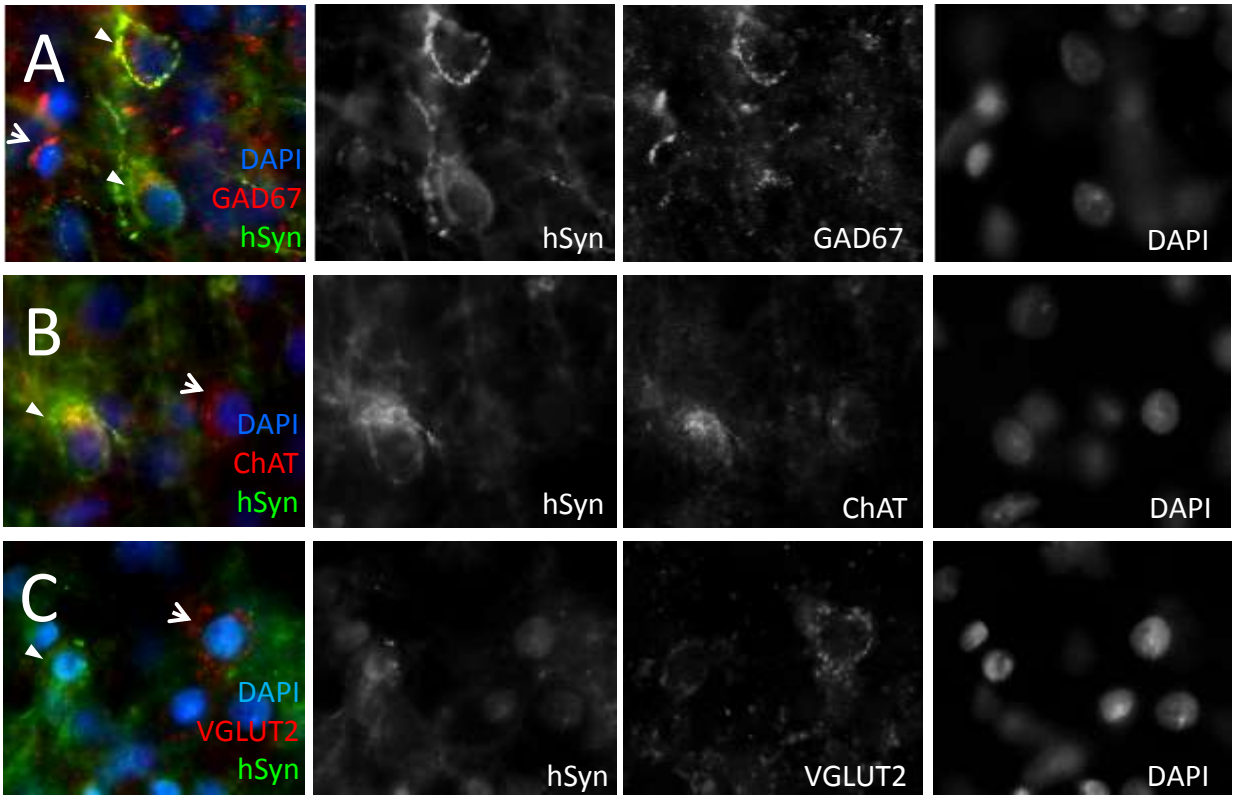


Figure 7 | hSynapsin LSIC-expressing cells colocalize with GAD67, ChAT, and VGLUT2 antibody stains.
Medial septal hSyn expressing neurons (green) are observed to colocalize with immunofluorescently labelled GABAergic neurons **(A)**, cholinergic neurons **(B)**, and glutamatergic neurons **(C)** in red. Colocalized cells are shown with solid arrowheads. Non-colocalized cells identified as GABAergic **(A)**, cholinergic **(B)**, or glutamatergic **(C)** are shown with arrows. DAPI nuclei stain is seen here in blue.

In order to quantify the specificity of the hSynapsin promoter, semi-quantitative cell counts were performed manually on GAD67, ChAT, and VGLUT2 stained septal tissue sections from hSyn infected animals. 6.25% of neurons expressing hSyn in ChAT-stained tissue were identified as cholinergic (Figure 8, light grey), whereas 68.3% of hSyn neurons in VGLUT2-stained tissue were identified as glutamatergic (Figure 8, dark grey) and 70.3% of hSyn neurons in GAD67-stained tissue were identified as GABAergic (Figure 8, black). The percentages of hSyn neurons identified as GABAergic and glutamatergic were significantly higher than the percentage of hSyn neurons identified as cholinergic (ANOVA: p-value < 0.05; 2 sample t-tests: p-values < 0.05).

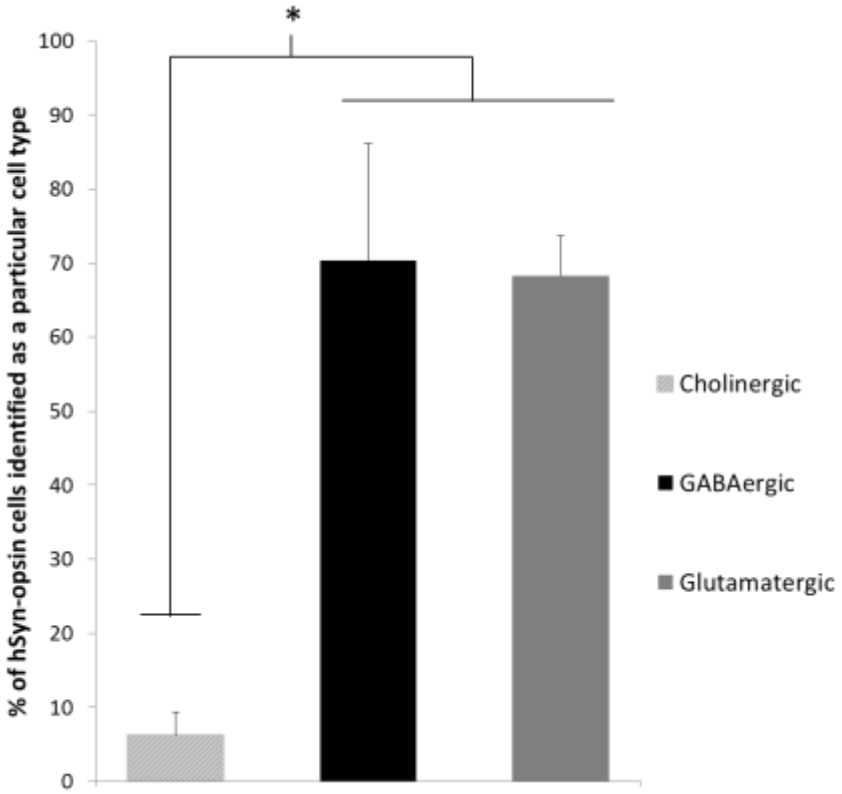


Figure 8| hSyn-opsin septal neurons are identified as GABAergic or glutamatergic at a significantly higher percentage than as cholinergic.
Average percentage of hSyn-opsin expressing cells which were identified as cholinergic (6.25%, **light grey**), GABAergic (70.3%, **black**), or glutamatergic (68.3%, **dark grey**) based on colocalized antibody staining. 3 way ANOVA: p-value < 0.05; 2 sample t-test between Cholinergic and GABAergic groups: p-value < 0.05; 2 sample t-test between Cholinergic and Glutamatergic groups: p-value << 0.05. Error bars represent SEM (SD/√n).

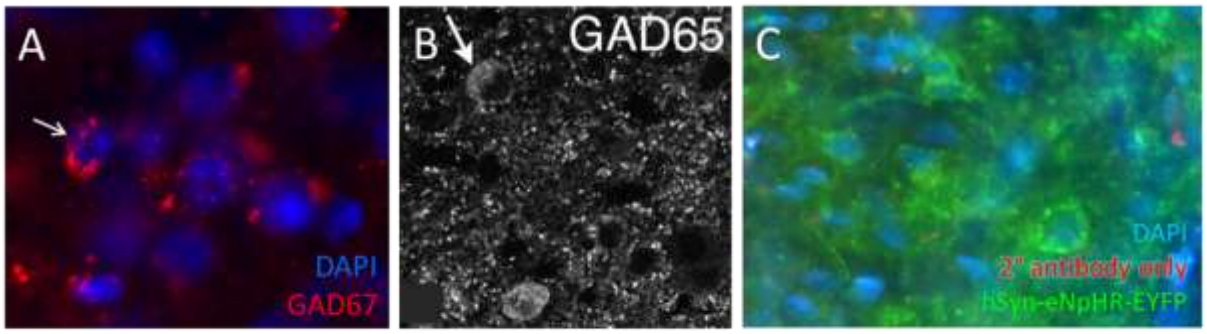
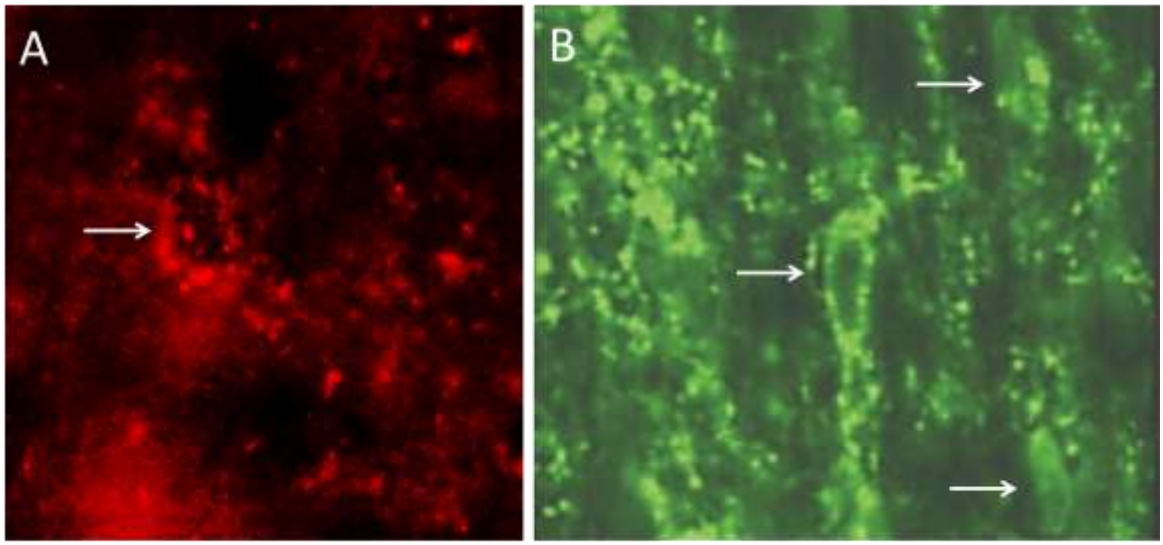


Figure 9| GAD67 antibody staining has a punctated appearance.
GAD67 antibody stain in the striatum **(A)** demarks GABAergic cells with a punctated appearance; GAD65 antibody staining in the septum by Luttgen et al (2005) **(B)** provides an example of how the stain is expected to look. Puncta are not observed when tissue is stained with red secondary antibody in the absence of primary antibody **(C)**. DAPI stain is seen in blue.

The puncta appearance of GAD67 staining is visualized in striatal neurons (Figure 9A), while no red puncta resembling staining are observed in the absence of the primary anti-GAD67 antibody (Figure 9C). Puncta appearance is corroborated by others and can be seen in an adapted figure from Luttgen et al (2005), showing similar staining with the GAD65 antibody (Figure 9B).

The VGLUT2 antibody also has a punctated appearance, visualized in the septal neurons (Figure 10A). Puncta appearance is corroborated by an adapted figure from Colom et al (2005), showing the same antibody stain (Figure 10B).



Adapted from Colom et al, 2005

Figure 10| VGLUT2 antibody staining has a punctated appearance.
VGLUT2 antibody stain in the medial septum **(A)** demarks glutamatergic cells with a punctated appearance; VGLUT2 antibody staining in the septum by Colom et al (2005) provides an example of how the stain is expected to look **(B)**.

PART II: C-fos Expression in the Medial Septum and Hippocampus as a Marker for Activation

Predictions of c-fos expression were made based on the connectivity between septal cell types and hippocampal pyramidal neurons (Figure 11). Glutamatergic septal neurons are excitatory and project directly to hippocampal pyramidal neurons. Cholinergic septal neurons, with the exception of a subtype which possess muscarinic Ach receptors, are excitatory and project directly to hippocampal pyramidal neurons. Stimulation of ChR2-expressing glutamatergic (Figure 11A, white) or cholinergic septal neurons (Figure 11A, grey) is thought to increase c-fos expression within the septum as well as within the hippocampal neurons they project to, while stimulation of NpHR-expressing glutamatergic (Figure 11B, white) or cholinergic septal neurons (Figure 11B, grey) is most likely to decrease c-fos expression within the septum as well as within the hippocampal neurons they project to. Inhibitory GABAergic septal neurons, in addition to projecting to local neurons of different phenotypes, project to an inhibitory GABAergic interneuron within the hippocampus, which projects to hippocampal pyramidal neurons. Stimulation of ChR2-expressing GABAergic septal neurons (Figure 11A, black) is hypothesized to increase c-fos expression within the septum, decrease activity of the interneuron, and increase c-fos expression within the hippocampal neurons they project to. Finally, stimulation of NpHR-expressing GABAergic septal neurons (Figure 11B, black) should lead to decreased c-fos expression within the NpHR MS neurons, increased c-fos within disinhibited local MS neurons, increased c-fos expression in the disinhibited GABAergic interneuron, and decreased c-fos expression within the hippocampal pyramidal neurons.

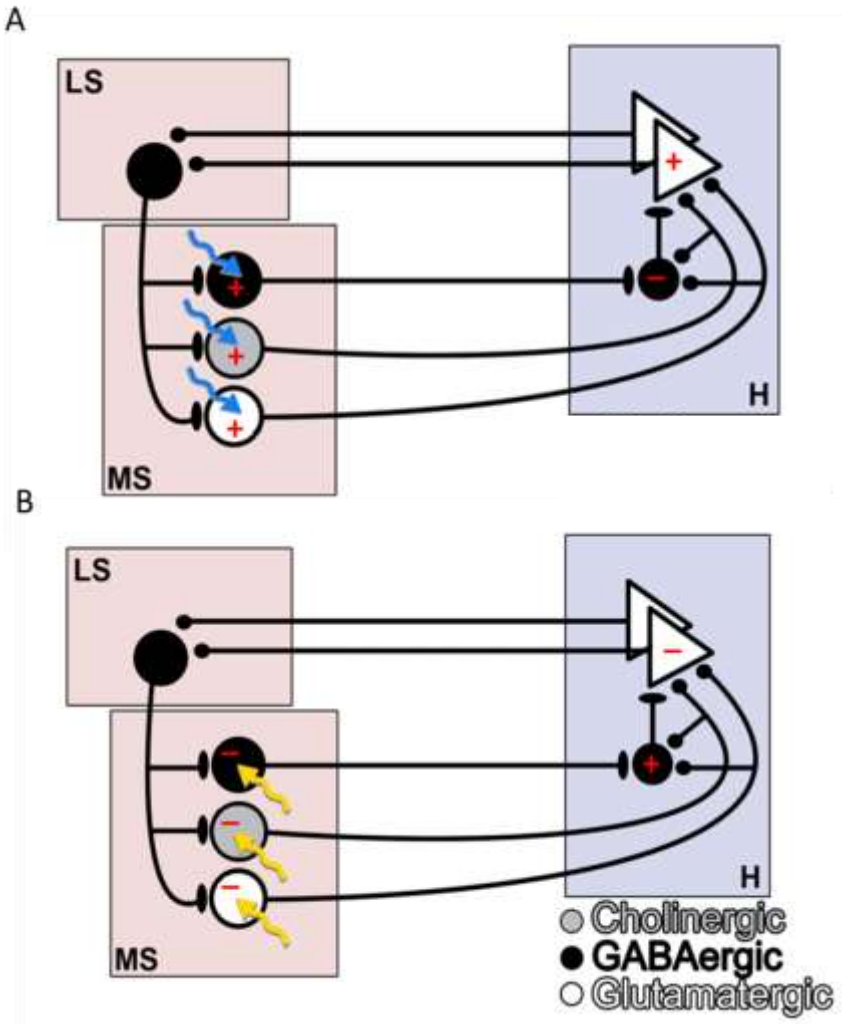


Figure 11 | Hypothesized outcomes of septal and hippocampal c-fos expression after stimulation of opsin-expressing cells in the MS.

A| Stimulation of glutamatergic (white) and cholinergic (grey) septal neurons expressing ChR2 is predicted to increase c-fos expression within the septal cells, as well as within the hippocampal pyramidal cells they project to. Stimulation of GABAergic (black) septal neurons expressing ChR2 is predicted to increase c-fos expression within the septal cells via inhibition of a GABAergic interneuron, disinhibiting the pyramidal cells, thus increasing c-fos expression in the hippocampus.

B| Stimulation of glutamatergic (white) and cholinergic (grey) septal neurons expressing NpHR is predicted to result in lower c-fos expression compared to baseline within the septal cells, as well as within the hippocampal pyramidal cells they project to. Stimulation of GABAergic (black) septal neurons expressing NpHR is predicted to result in lower c-fos expression compared to baseline within the septal cells, increasing activity of the interneuron, and inhibiting the pyramidal cells, resulting in a lower c-fos expression in the hippocampus compared to baseline.

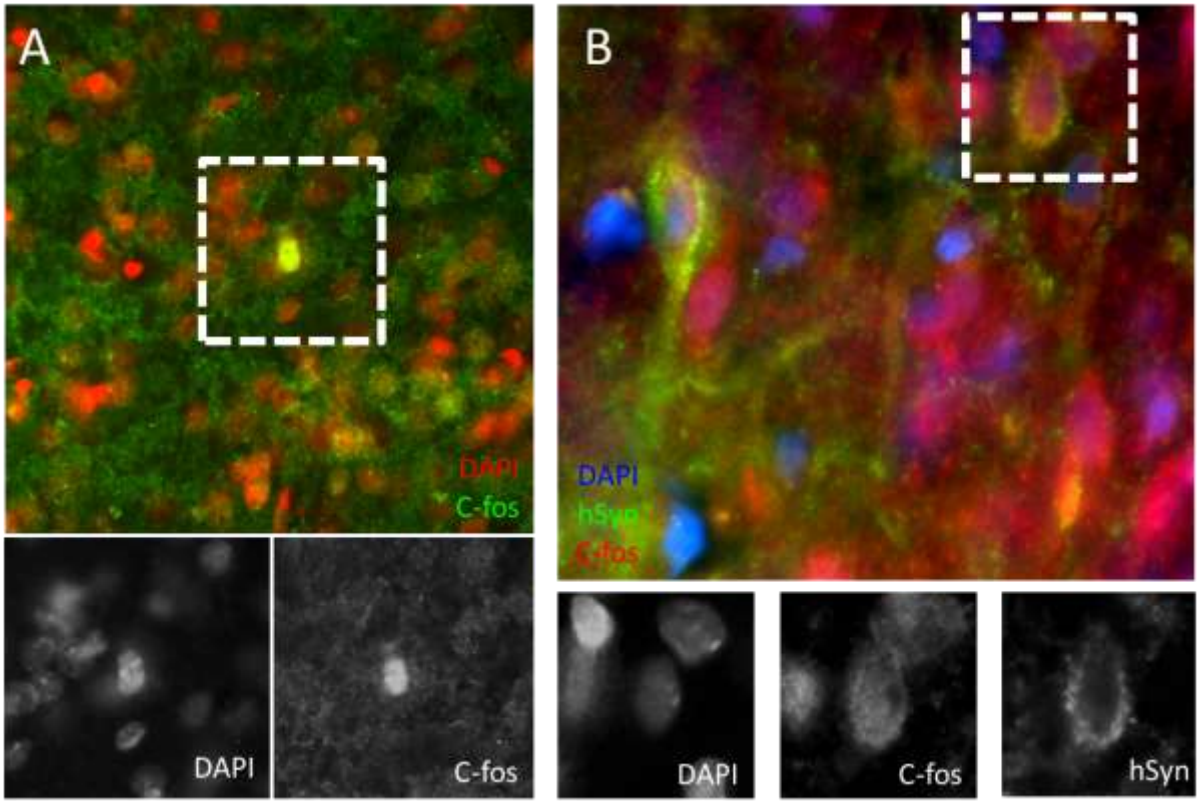


Figure 12 | C-fos positive cell observed in the hippocampus of a CaMKIIα-ChR2 animal.
A| C-fos (green) colocalized with a hippocampal nucleus (red) in a CaMKIIα-ChR2 animal. DAPI nuclear stain seen in red. **B|** Non-nuclear c-fos staining (red) in a medial septum hSyn-ChR2 expressing cell. DAPI nuclear stain seen in blue.

C-fos positive neurons were observed in the hippocampus of 1 stimulated CaMKIIα-ChR2 animal (Figure 12A) and 1 ChAT-CRE animal (Figure 13A) as predicted, although neurons were extremely sparse. No such neurons were observed in the medial septum of the same animals, as was the case for all the stimulated animals. While red staining is visible within the CaMKIIα septum (Figure 12B), the stain is not bright or localized to the nucleus, suggesting detection of non-translocated c-fos as would be expected in a non-stimulated cell. However, many c-fos positive neurons, with nuclear c-fos localization, were observed in the lateral septum

(Figure 13B) and bed nucleus of the stria terminalis (BNST) (Figure 13C) of the ChAT-CRE animal.

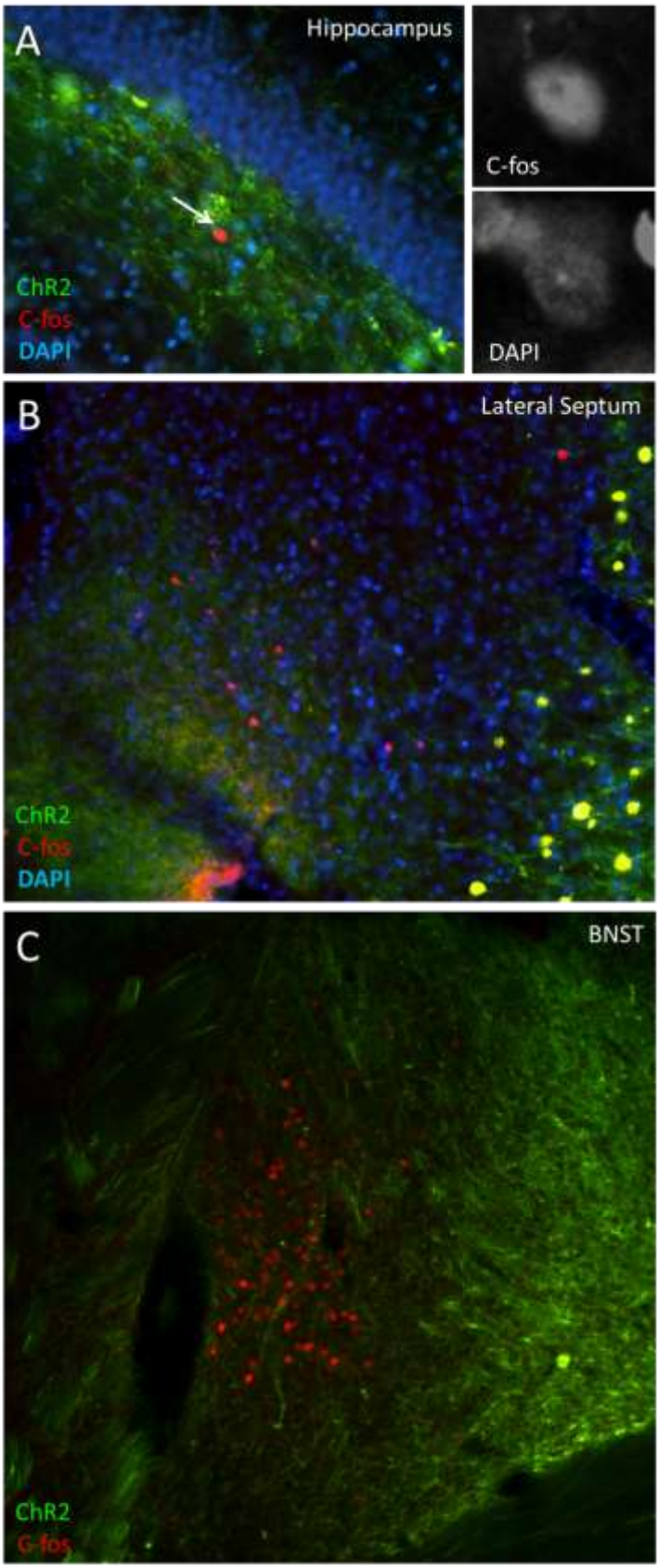


Figure 13 | Cells positive for C-fos are observed in a ChAT-CRE ChR2 animal.
C-fos positive cells (red) were observed in the hippocampus **(A)**, lateral septum **(B)**, and bed nucleus of the stria terminalis (BNST) **(C)**, of a ChAT-CRE animal expressing ChR2. C-fos positive cells were not observed in the medial septum. Cells coexpressing ChR2 and c-fos were also not observed. DAPI stain seen here in blue.

In order to test whether the length of the stimulation paradigm was influencing c-fos expression at the stimulated site, mice injected with Thy1-ChR2 in the prefrontal cortex (as per the procedure by Kumar et al, 2013) were stimulated for either 5 or 90 minutes at the midline of the region. In the animal stimulated for 5 minutes (Figure 14A), c-fos positive neurons were observed in various regions outside of the stimulated area, such as in the dorsal, lateral, and mid-dorsal frontal cortex. In the animal stimulated for 90 minutes (Figure 14B)**,** c-fos positive neurons were observed mainly above the stimulated region, in the dorsal frontal cortex.

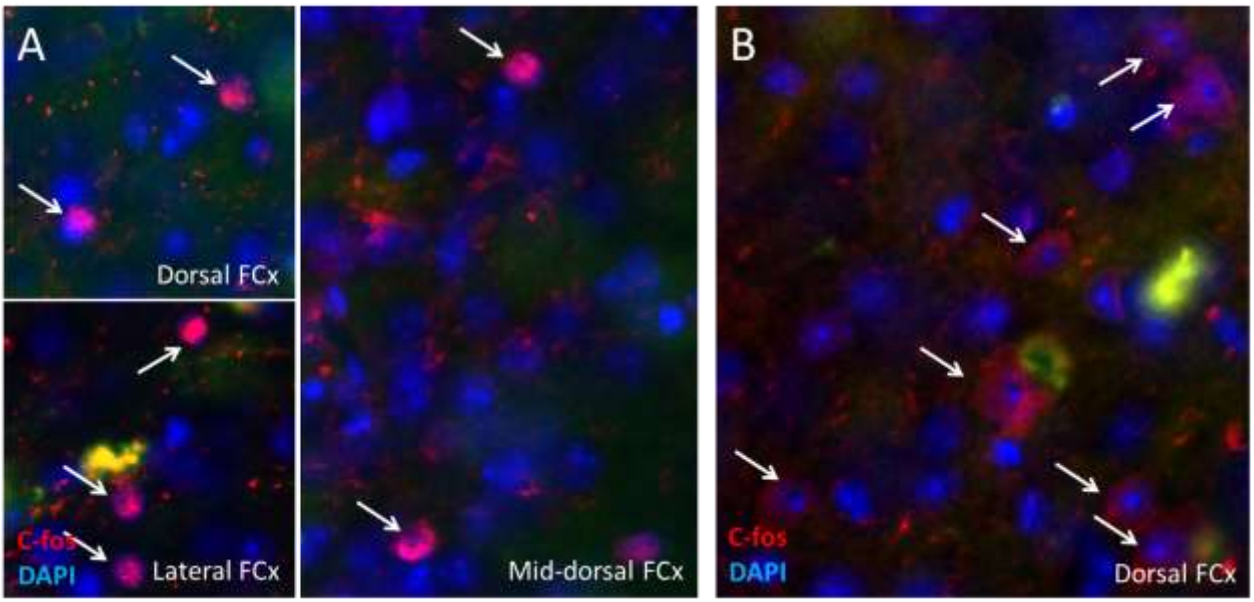


Figure 14 | C-fos positive cells observed outside of the stimulated region in Thyl-ChR2 mice receiving different stimulation paradigms.
After 5 minute **(A)** or 90 minute **(B)** optogenetic stimulation at the midline of the prefrontal cortex, where Thyl-ChR2 expressing cells were observed, c-fos positive cells were found outside of this region. In the animal receiving 5 minutes of stimulation **(A)**, c-fos positive cells were found above the stimulated region (dorsal frontal and mid-dorsal frontal cortex), as well as lateral to the stimulated region (lateral frontal cortex). In the animal receiving 90 minutes of stimulation **(B)**, c-fos positive cells were found mostly above the stimulated region (dorsal frontal cortex).

DISCUSSION

PART I: Immunohistochemical identification of opsin-expressing cells and promoter specificity to cell-types

To summarize the results of Part I:

- Cholinergic MS neurons can be targeted using ChAT-CRE transgenic animals.
- Glutamatergic MS neurons can be targeted using the CaMKIIα promoter sequence.
- The hSyn promoter sequence may be specific for GABAergic and certain glutamatergic MS neurons, but not cholinergic neurons.

A number of experiments have singled out medial septal cholinergic neurons for optogenetic experiments through the use of transgenic animals: Vandecasteele et al (2014) and Yi et al (2015) have both used ChAT-CRE transgenic animals and found double-floxed inverted open reading frame (DIO) opsins to be expressed specifically within the cholinergic neurons of the medial septum and hippocampus, respectively. Our result that DIO-ChR2 expression colocalizes with anti-ChAT antibody in the medial septum of transgenic ChAT-CRE animals (Figure 5) supports the current literature findings that transgenic animals allow for specific targeting of the cholinergic neuron population.

The glutamatergic neurons of the medial septum are a relatively new discovery; in comparison with their cholinergic and GABAergic counterparts, very little research has been done exploring a method to specifically target them for activation or inhibition. Although the CaMKIIα promoter has been shown to be selective for glutamatergic neurons in the hippocampus, our finding that the CaMKIIα promoter allows for expression of opsins

exclusively in the glutamatergic neurons of the MS is a new addition to the optogenetic field. This finding will allow for future selective manipulation of this population, which will further the investigation of the effects that glutamatergic neurons exert on the hippocampus.

Selective targeting and expression of opsins in GABAergic neurons of the medial septum was hypothesized to be achieved through the use of the hSynapsin promoter. This prediction was motivated by the work of Kaifosh and colleagues (2013), whose work revealed three findings: (1) almost no ChAT immunoreactivity was found in the large population of hSyn-expressing medial septal neurons, (2) stimulating hSyn-ChR2 medial septal neurons led to a GABAergic response in the CA1 layer of the hippocampus, and (3) treatment with gabazine, a GABAergic inhibitor, completely abolished this GABAergic response in the hippocampus. We have observed that hSyn-ChR2-EYFP and hSyn-eNpHR3.0-EYFP colocalize with GABAergic and glutamatergic neurons in the septum, as well as with cholinergic neurons. In order to investigate whether the hSyn-opsin expressing neurons were of a particular phenotype, the number of colocalized neurons was calculated as a percentage of hSyn-expressing neurons in three animals. Out of the total number of identified hSyn neurons in ChAT, GAD67, and VGLUT2 stained sections of the medial septum, an average of 6.25% were identified as cholinergic, 70.3% were identified as GABAergic, and 68.3% were identified as glutamatergic. Since the sum of these values far exceeds 100%, it is possible that some septal neurons express more than 1 type of neurotransmitter (i.e., neurons positive for both GAD67 and VGLUT).

In support of this theory, it has been found by Sotty et al (2003) that approximately 60% of MS/DBB neurons that are GAD67 positive also exhibit one or both of the VGLUT stains, while 44% of the GAD67 positive population also appears to exhibit ChAT immunoreactivity. Additionally, Colom et al (2005) discovered that approximately 25% of glutamatergic labeled

septal neurons are also labeled with ChAT, GAD67, or both antibody stains; specifically, 14% of glutamatergic labeled septal neurons are also identified with the GAD67 stain. Close examination of the triple-stained images published by Colom et al reveals punctated overlapped staining across the three antibodies.

Additionally, neurons throughout the brain have been discovered which release more than 1 type of neurotransmitter: VTA neurons which project to the lateral habenula are positive for both GAD and VGLUT2, and their post-synaptic current patterns suggest that they release both GABA and glutamate neurotransmitters (Uchida, 2014). Basal forebrain cholinergic neurons are found to release glutamate as well (Allen et al., 2006). Post-synaptic current analysis of MS/DBB neurons in particular has made evident the presence of septal neurons co-releasing acetylcholine and glutamate, GABA and glutamate (Huh et al., 2008), and acetylcholine and GABA (Saunders et al, 2015).

Another possible explanation for why there may be an overestimation of hSyn neurons which are GABAergic or glutamatergic might stem from a technical difficulty in accurately identifying positive neurons. Given the punctated distribution of the GAD67 and VGLUT2 antibody stains, it is unclear at times whether the observed puncta are within the limit of the neuronal soma or adjacent to the cell. This issue has been reported by others. One reason offered by Colom et al (2005) is that extensive synaptic terminal contacts around a soma in the medial septum, such as cholinergic terminals in association with a glutamatergic soma, may appear as a positively stained cholinergic neuron. This is more likely to be a confounding variable when identifying GABAergic neurons with surrounding glutamatergic synaptic contacts or vice versa, due to the regularly observed punctated appearance of both the GAD (Lüttgen et al., 2005) and VGLUT (Colom et al, 2005) antibody stains (Figures 9 and 10).

While overlapped stain appearance is corroborated by other experiments (Sotty et al, 2003; Colom et al, 2005), there may be other explanations which exist to support the expression of hSyn-opsins in more than one cell type in the MS. Kaifosh et al (2013) found that septal neurons expressing hSyn-ChR2 exhibited almost no ChAT immunoreactivity, and that when GABAergic transmission was blocked during stimulation of these neurons, the hippocampal response in GABAergic neurons was entirely blocked; this suggested that hSyn septal targets mediate only a GABAergic hippocampal effect. Our finding that hSyn septal targets may include glutamatergic and GABAergic neurons does not necessarily go against this result. Glutamatergic septal neurons project directly to the glutamatergic neurons of the hippocampus, as well as to GABAergic, glutamatergic, and cholinergic interneurons within the MS/DBB itself (Leão et al., 2015); in fact, Leão et al found that 10% of identified glutamatergic septal neurons projected to a GABAergic postsynaptic neuron within the MS/DBB. Hajszan et al (2004) examined VGLUT2 boutons of the MS/DBB region and found a great number of synaptic associations between these glutamatergic boutons and septohippocampal parvalbumin neurons, which are a subset of GABAergic neurons (Lüttgen et al., 2005). Huh et al (2010) have found that neurons which exhibit VGLUT2 solely release glutamate neurotransmitter. As our study utilized anti-VGLUT2 antibody, it is very likely that hSyn-expressing GABAergic neurons were misidentified as glutamatergic due to surrounding VGLUT2-positive synaptic contacts. Thus, while hSyn may be expressed in septal neuron populations other than the GABAergic population, such as glutamatergic neurons, the projections of these targeted neurons to septohippocampal GABAergic interneurons could still mediate the entirely “GABAergic effect” (Kaifosh et al, 2013) observed in the hippocampus.

PART II: C-fos expression as a marker for optogenetic activation

To summarize the results of Part II:

- C-fos expression is not observed in the stimulated region.
- Limited c-fos expression is observed in output regions of the MS.
- C-fos expression in the BNST was observed in 1 ChAT-CRE ChR2 animal.
- Changing stimulation paradigm has had inconclusive results.
- Visualization of c-fos expression may be affected by external variables.
- C-fos expression itself may be affected by external variables.

Optogenetic stimulation has been shown to activate neurons expressing excitatory opsins (Krook-Magnuson et al, 2013). Based on our predictions (Figure 11), c-fos expression should have been extensive in the medial septum. It remains unclear as to why our many different optogenetic stimulation experiments were unable to induce strong c-fos expression within the stimulated region, whether that region was the medial septum or the prefrontal cortex.

It is interesting that in multiple ChR2-expressing animals, stimulating in the MS resulted in sparse hippocampal c-fos staining with no septal staining. It has been shown that stimulation of neurons in one region can lead to neuronal activation and c-fos expression in the target output region (Kumar et al, 2013). Since the hippocampus is the main target output of the stimulated septal neurons, it was predicted that this target region would express c-fos. Additionally, while the MS was not c-fos positive, many other regions either related to the MS, such as the lateral septum, or unrelated to the MS, such as the bed nucleus of the stria terminalis (BNST), showed numerous positive c-fos neurons. The lateral septum, composed mainly of GABAergic neurons, projects to and receives glutamatergic inputs from both the medial septum and the hippocampus

(Risold & Swanson, 1997). The BNST receives input mostly from the amygdala (Weller & Smith, 1982), and projects mostly to the stria terminalis, stria medullaris, and medial forebrain (Swanson & Cowan, 1979). Thus, it is conceivable that c-fos expression in the lateral septum is a result of MS optogenetic stimulation. It is unclear whether the strong c-fos expression observed in the BNST, is a far-off effect of activation in the MS, an effect of the spread of the viral vector or light, or a reflection of the general state of the animal during stimulation.

There are a few possible variables which could be affecting our ability to visualize c-fos expression. One variable is quality of perfusion of the animal. Our protocol included prolonged submersion of the animal's head in paraformaldehyde (PF) following the perfusion. This was done to allow the tissue to properly fix before attempting to remove the optical headpiece so that the ferrule placement could be localized. However, the extended exposure to PF could be masking the target antigen of the primary antibody (Rhodes & Trimmer, 2006). This theory is partially supported by the very strong c-fos positive staining, albeit in the non-stimulated region, found in the animal that was perfusion fixed with no overnight post fixation (Figure 13). This animal also served as a positive control for c-fos antibody staining.

Another variable which could be affecting c-fos visualization is stimulation paradigm. Our 90 minute stimulation length stems from prior electrical stimulation experiments, in which this is the norm, and other experiments have corroborated this paradigm (Krook-Magnuson et al, 2013). However, successful c-fos experiments have also been performed in which the duration of optogenetic stimulation is far less than 90 minutes (Jones et al., 2015; Kumar et al., 2013; Liu et al., 2012) or in which the interval between light pulses is on the scale of seconds or minutes, rather than milliseconds (Liu et al., 2012; Schoenenberger et al., 2009). To test whether stimulation length had an effect on c-fos expression, we performed an additional experiment in

which mice were injected with Thy1-Chr2 in the prefrontal cortex and optically stimulated at the midline of that region at maximum intensity, with either 10ms pulses at 4 Hz for 5 minutes or 10ms pulses at 35 Hz for 90 minutes. This procedure was adapted from that of Kumar et al (2013). C-fos expression was visible in both brains in areas surrounding the stimulated region, as specified before (Figure 14). C-fos expression was observed to be more widespread in the 90 minute brain. For subsequent repetitions of this trial, stimulation will be confined to one side of the prefrontal cortex in order to use the non-stimulated side as a control.

CONCLUSIONS

Cholinergic and glutamatergic neurons of the medial septum can selectively be targeted to express opsins by using ChAT-CRE transgenic animals and the CaMKIIα promoter, respectively. The finding that the CaMKIIα promoter may be used to specifically target glutamatergic neurons in the MS is a new addition to the optogenetic field and allows for future manipulation of this cell type. It may be of interest to further explore the specificity of the CaMKIIα promoter by quantifying the cell phenotypes which express CaMKIIα. GABAergic neurons of the medial septum can be targeted to express opsins using the hSyn promoter; however, this promoter does not exclusively target GABAergic neurons. While stimulating hSyn-opsin septal neurons has been found to elicit a purely GABAergic hippocampal response (Kaifosh et al, 2013), it does not necessarily mandate that the septal targets be entirely GABAergic themselves, since glutamatergic neurons have been observed to project to GABAergic neurons within the MS/DBB.

C-fos expression can be used as a marker for activated cells. While we have observed c-fos expression in the hippocampus, lateral septum, and BNST of animals stimulated in the medial septum, we remain unable to visualize c-fos expression within the optogenetically stimulated area. Preliminary studies have explored possible variables that could affect visualization of c-fos expression, including perfusion and fixation quality, stimulation length, and length of interval between light pulses.

SUPPLEMENTAL INFORMATION

Animals:

DRAG11: control hSyn-EYFP (no LSIC, received stim)

DRAG16: control DIO-hChR2-mcherry (no stim)

DRAG23: control CamKIIa-hChR2-mcherry (no stim)

DRAG20, 21, 22: hSyn-eNpHR3.0-EYFP

DRAG12: hSyn-hChR2-EFYP

DRAG19: CamKIIa-eNpHR3.0-EYFP

DRAG15: DIO-hChR2-mcherry

Staining procedure for ChAT/GAD67/VGLUT/c-fos:

Blocking/permeabilization: 4% Normal Donkey Serum, 0.1% Triton-X, PBS
Primary antibody stain: 2% Normal Donkey Serum, Primary antibody at appropriate concentration (see below), PBS

Primary Antibody	**Source**	**Concentration for use**
C-Fos	Rabbit	1:500
GAD67	Mouse	1:1000
VGLUT	Mouse	1:1000
ChAT	Goat	1:500

Rinse 3x in PBS at 10 minutes each.
Secondary antibody stain: 2% Normal Donkey Serum, Donkey-anti-source Secondary antibody at 1:1000 concentration, PBS.
Rinse 3x in PBS at 10 minutes each.

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