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# LOCALIZATION OF SYNAPTIC SPECIFICITY GENE KIRREL3 IN THE HIPPOCAMPUS

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## ABSTRACT

Synaptic specificity is the ability for neurons to form synapses with specific post synaptic partners. The hippocampus is a brain region involved in learning and memory and is a great model for understanding synaptic specificity. Kirrel3 is a synaptic specificity gene and mutations in the Kirrel3 gene are found in neurodevelopmental diseases such as autism and intellectual disability. Mice lacking Kirrel3 have an excitation-inhibition imbalance in the hippocampus. Within this region, Kirrel3 is expressed in two distinct cell types; DG and GABAergic interneurons, which are connected within a complex circuit called the mossy fiber synapse. While we know that Kirrel3 is expressed in DG and GABAergic interneurons, we do not know if Kirrel3 is localized to these synaptic structures or how it acts to facilitate synapse formation. Determining the localization of Kirrel3 is important as it will give more insight to the mechanism of Kirrel3 and how certain mutations in the gene may lead to neurodevelopmental disorders. We hypothesized that Kirrel3 localizes to mossy fiber filopodial synapses along the DG axon. We investigated Kirrel3 localization by introducing an epitope-tagged Kirrel3 *in vitro* and *in vivo* via an adeno-associated virus

(AAV). We then immunostained for the epitope tag and examined its localization by confocal microscopy to determine if Kirrel3 localizes at filopodial synapses along DG axons. Our results indicated that Kirrel3 *in vitro* localizes to dendritic shafts and cell bodies. These results also found that the use of an AAV is an effective method of introducing and expressing a packaged Kirrel3 gene into cells. We found that Kirrel3 *in vivo* localizes to the dendritic shafts and cell bodies of DG neurons in the hippocampus. We propose that Kirrel3's localization to DG dendrites *in vivo* could be due to post-translational regulation of Kirrel3, undetectable epitope signal in the mossy fiber synapses caused by protein-protein interactions, or because this is the true area of localization of the protein.

# INTRODUCTION

The brain is composed of a complex network of cells called neurons that work together to interpret sensory information from the environment, learn, drive behavior, and ultimately give rise to consciousness. Neurons send information to other neurons via connections called synapses. Synapses do not form randomly between neurons, but rather select specific targets amongst a dense environment of incorrect targets (Williams et al, 2010). This process, termed synaptic specificity, is the ability for neurons to form specific synapses with other neurons (Williams et al, 2010).

The hippocampus, a brain region involved in learning and memory, is an excellent model to study synaptic specificity because the hippocampus is a highly laminar structure, in which different layers house relatively pure populations of distinct synapse types. Three distinct regions of the hippocampus defined by their principal glutamatergic neuron types are the Dentate Gyrus (DG), the CA3, and the CA1. There are also GABAergic interneurons in each region. One particular synapse that shows a high degree of synapse specificity is the mossy fiber synapse. Here, the DG axons synapse with GABAergic interneurons and CA3 neurons via the mossy fiber synapse. The DG mossy fiber has a large main bouton that synapses with CA3 neurons and filopodia that extend out from the main bouton and synapse with GABA dendrites (Figure 1). Mossy fibers synapse more frequently with inhibitory GABAergic interneurons than with excitatory CA3 pyramidal neurons (Acsady et al, 1998). This circuit of DG-GABA-CA3 allows for regulation of the relative excitation and inhibition of CA3 neurons. The main bouton directly excites the CA3 neuron while filopodial synapses excite nearby

GABAergic interneurons, which then inhibit CA3 neurons via feed-forward inhibition (Figure 1). Excitation-inhibition balance is important so that the CA3 neurons do not get overexcited (Torborg et al, 2010). Rett syndrome is an example of a disease that occurs in the hippocampus due to improper excitation-inhibition balance. Autism is another disease that likely involves an imbalance of excitation and inhibition in the hippocampus, with studies reporting too much excitation in the autistic brain (Baroncelli et al, 2011).

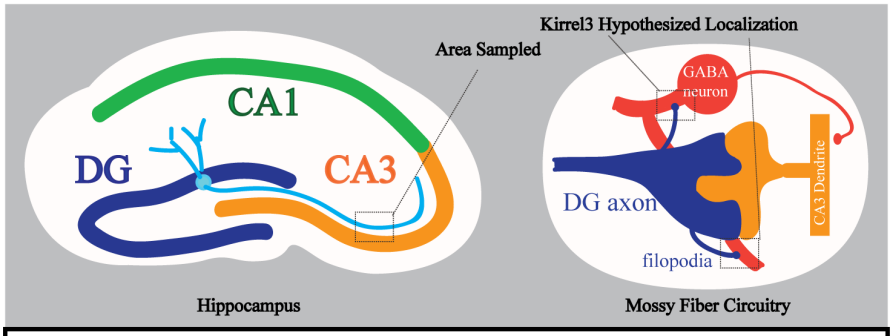


Figure 1. This diagram depicts the mossy fiber synaptic complex circuitry. (Left) cross section of the hippocampus showing the DG and CA3 subregions. (Right) sampled area showing the microcircuit. The DG axon synapses with the CA3 dendrite via the large main bouton. Coming off the DG axon are filopodia, which form synapses with GABAergic interneurons. The GABAergic interneurons then synapse onto CA3s to provide feedforward inhibition.

The formation of synapses is a process that requires recruitment of many different factors for proper function. This includes vesicles for neurotransmitters and homophilic binding proteins that act as cell adhesion molecules. Each synapse type has a unique set of factors required for proper specificity. This is proven by the protein neurexin that has various different splice forms used in different synapse types. Cell adhesion molecules are an essential part of proper synaptic functioning. Cell adhesion

molecules are located on the surface of cells and function by binding to cell adhesion molecules on nearby cells. These proteins link to each other via domains located on the extracellular domain of these proteins. In synapses, cell adhesion molecules are homophilic and presynaptic cell adhesion molecules bind a copy of itself located on the post-synaptic cell. These interactions help cells stick together and allow neurons to recognize their synaptic partners.

The gene Kirrel3, is a homophilic binding protein that regulates mossy fiber synapse development within the hippocampus (Martin et al, 2015). Kirrel3 is a transmembrane protein that contains 5 immunoglobulin domains on the extracellular portion of the protein (Martin et al, 2015). The intracellular domain has a PDZ binding region which may bind a protein known as CASK (Gerke et al, 2006). Kirrel3 has been shown to be synaptically localized by various methods. First, Kirrel3 was found to be in the synaptosomes, which is an isolated synapse. Next, Kirrel3 localized *in vitro* alongside post-synaptic markers in wild type cells (cells with Kirrel3) but did not in cells that had the Kirrel3 gene knocked out. Lastly, Kirrel3 clusters at cell junctions. These three pieces of evidence suggest that Kirrel3 is a synaptic cell adhesion molecule.

Interestingly, Kirrel3 has is associated with various neurological diseases. A translocation in the chromosome that contains the Kirrel3 gene is thought to lead to intellectual disability among humans (Bhalla et al, 2008). A 2.899 mega base interstitial deletion on the chromosome 11 of the human genome, which deletes part of the Kirrel3 gene, has been found to play a role in Jacobsen's syndrome (Guerin et al, 2012). A recent study found that Kirrel3 knockout mice exhibited abnormal social behaviors

related to neurodevelopmental disorders (Hisaoka et al, 2018). Kirrel3 is also a hotspot gene that is implicated in autism spectrum disorder (Michaelson et al, 2012) (Neale et al, 2012) (Liu et al, 2015). The specific R205Q mutation in the Kirrel3 gene is one that has been linked specifically to autism. The R205Q point mutant is a mutation on the extracellular domain that changes an arginine residue to a glutamine. It is hypothesized that this mutation on the extracellular domain affects homophilic binding between presynaptic and postsynaptic Kirrel3 molecules in the mossy fiber synapse. This leads to loss of synaptic specificity, which leads to overexcitation of the CA3 neurons in the hippocampus.

Through the use of *in situ* hybridization studies, the mRNA of Kirrel3 was shown to express in the DG neurons of the hippocampus and in GABAergic interneurons in the hippocampus (Martin et al, 2015). This finding suggests that Kirrel3 acts within the DG neurons and interneurons. The protein plays a vital role in the regulation of the mossy fiber synapses that are found between these neurons. Mossy fiber synapses were analyzed in wild-type and Kirrel3 knockout mice. The results found that the knockout mice had fewer mossy fiber filopodia than the wild-type suggesting that Kirrel3 plays a role in the development of mossy fiber filopodia (Martin 2015). Kirrel3 also plays a strong role in excitation-inhibition within CA3 neurons. Wild type neurons and Kirrel3 knockout neurons were studied and their excitation/inhibition ratios were measured. Knockout neurons had higher excitation than wild type. Kirrel3 likely allows for more inhibition since knocking out the gene causes overexcitation of the CA3 neurons. When Kirrel3 is not present, mossy fiber filopodial synapses between the DG neurons and

GABAergic interneurons are likely not formed. This leads to lower amounts of mossy fiber filopodia and overexcitation of the CA3 neurons. The defects in mossy fiber synapses found in Kirrel3 mice last through to adulthood. There are generally less mossy fiber filopodia in adult mice than in younger ones but the difference in filopodia is still significant between adult wild type and Kirrel3 knockout mice (Martin et al, 2015).

Importantly, mice during development have three distinct mossy fiber synapse states. The first is when the mossy fiber filopodia lack any synaptic vesicles nearby nor any post synaptic densities and are described as synapse-free. The second has clusters of synaptic vesicles but no post synaptic densities which are considered partial synapses. The third has synaptic vesicle clusters near a post synaptic density and these synapses are considered “complete synapses”. Knockout Kirrel3 has been found to have more synapse free filopodia and a lower number of synapses that are partial or complete suggesting that Kirrel3 plays an important role in regulating the formation of the mossy fiber filopodial synapses (Martin et al, 2017). Kirrel3 knockout mice form less mossy fiber filopodial synapses with GABAergic interneurons. The morphology of the filopodia in these synapses, however, are unaffected when Kirrel3 is knocked out. This suggests that Kirrel3 regulates the formation of these filopodial synapses with GABA neurons but does not play a role in affecting the morphology of the either the main bouton or the filopodia themselves (Martin et al, 2017). The overall implications of these findings indicate the nature of Kirrel3 in synaptic development and how the lack of the protein plays a noticeable effect in the development of mossy fiber filopodial synapses.

While we currently know that Kirrel3 regulates the formation of mossy fiber filopodia, we do not know the where the protein exactly localizes in the hippocampus. Knowing this is crucial as it can help explain its mechanism of action and how mutations in the protein may cause the diseases that are associated with mutations in the Kirrel3 gene. Furthermore, no current studies in the field of neuroscience have examined localization of transmembrane proteins such as Kirrel3 in neurons. This makes the task challenging as we have to design our own tools and methods to determine Kirrel3 localization. Determining the localization will make future localization studies for transmembrane synaptic proteins a lot easier.

From *in situ* hybridization studies, the mRNA of Kirrel3 is expressed in the DG and GABAergic interneurons and importantly not the CA3 neurons. The DG and GABAergic interneurons are also where mossy fiber filopodia form synapses. Furthermore, Kirrel3 knockouts have impaired filopodial development at these synapses, suggesting that Kirrel3 is important in maintaining the development and growth of the mossy fiber filopodial synapses. For these reasons, we hypothesize that Kirrel3 will localize to the mossy fiber filopodia between the DG and GABA neurons (Figure 1). We will test our hypothesis by infecting an AAV containing the Kirrel3 gene and an epitope tag into mice hippocampi, and determine its localization by antibody immunostaining and confocal microscopy.

# METHODS

Determining the localization of Kirrel3 requires multiple steps. The general workflow involves cloning an expression vector containing the Kirrel3 gene and an epitope tag for detection, packaging this plasmid into a virus, and injecting the virus into CD1 mice and harvesting the hippocampal tissue for analysis (Figure 2).

## Cloning Plasmids

First, an expression vector encoding the Kirrel3 protein was cloned. An expression vector is essentially a piece of circular bacterial DNA that has the gene for Kirrel3 inserted into it. Bacterial DNA is circular shaped and are called plasmids. In an expression vector, there are multiple important regions that play a role in transcribing the DNA to messenger RNA. First, the gene being inserted to a plasmid is crucial. The gene we used is a construct known as GFP-2A-3xHA-Kirrel3 (Figure 3D). This construct is polycistronic meaning that the gene codes for the production of more than one polypeptide. The GFP protein acts as a cell filler and allows for visualization of cell morphology when viewing under fluorescence in a microscope. This is essential as we need to be able to see what part of the cell we are examining. Next is the 2A peptide, which is a peptide that can link two genes (Kirrel3 and GFP) and self-cleaves itself post translationally. This is essential as it allows us to insert a cell filler and our gene of interest in the same construct. Thus, every cell that contains GFP must contain Kirrel3 as well. The alternative to this method involves co-transducing a cell filler virus along with a virus containing our gene of interest. This method is more difficult as different cells may get different viruses and it is hard to tell which cells received which virus. Using the

2A peptide construct, we know that if a cell got transduced with the cell filler, then it must contain the Kirrel3 protein. The Kirrel3 gene itself must be part of the gene insert as it is the protein we are analyzing for localization. The Kirrel3 gene has an epitope tag attached to the extracellular domain. Currently, there are no useful antibodies that can recognize Kirrel3. To get around this, the epitope tag is used as there are effective antibodies that can recognize the epitope and if we see epitope fluorescence we know it is from the same protein as Kirrel3. Antibodies are important as they are required for inducing fluorescence of various proteins as discussed later.

Next, the promoter region of a plasmid is also essential. The promoter region of a gene is what initiates transcription of DNA to mRNA. RNA polymerase binds the promoter sequence and then cruises down the DNA and produces and RNA transcript. There are two strands of DNA, the sense strand and antisense strand. The RNA transcript is the exact same as the sense strand except thymine bases are replaced with uracil. The promoter in a plasmid is important as certain sequences can be stronger or weaker or be more active earlier or later in the development of a cell. Promoters can also be specific to certain cell populations. We used the CamKII promoter sequence. This sequence is derived from the promoter for a protein called calmodulin dependent kinase II, that functions in excitatory neurons. This sequence is known to be on the weaker side, but becomes stronger later in the development in the cell. Furthermore, this promoter sequence was small enough to allow us to package the full construct into a virus.

The inverted terminal repeat (ITR) segment and the WPRE segment of a plasmid are essential for packaging the plasmid in a virus. The ITR region is in a plasmid as it allows for efficient packaging of the plasmid into a viral vector. The virus we are using is the adeno-associated virus (AAV) and the ITR region is an essential element for AAV packaging of DNA. The WPRE element enhances expression of the gene when delivered in a virus. Furthermore, each mRNA transcript has a polyadenylated tail, which stabilizes the transcript in the cell. This is also an important feature of a plasmid so that the mRNA remains stable before being translated.

In order to clone the GFP-2A-3xHA-Kirrel3 gene we needed to use two plasmids and insert one into the other. The first plasmid was the Kirrel3 pBOS vector. This plasmid contained Kirrel3 under a EF1 promoter, but did not contain the elements required for packaging into an AAV. The second plasmid was the plasmid containing the CamKII promoter, ITR regions, and other AAV elements, but not Kirrel3. Restriction enzymes were used to cut Kirrel3 from pBOS and to cut open the CamKII AAV vector and paste Kirrel3 into our desired plasmid. We found restriction sites on our CamKII plasmid and needed to insert this restriction enzyme sequence into our Kirrel3 plasmid. The restriction enzymes we used were HindIII and Xho1, both of which produce sticky ends. Our first step required polymerase chain reaction (PCR) to add a restriction enzyme sequence to the Kirrel3 vector. PCR amplifies segments of DNA. The first step in PCR is to design primers that the DNA polymerase can bind to and replicate the DNA. Usually there are forward and reverse primers between two segments of DNA that polymerases bind to and amplify. Primers must be complementary to the DNA sequence they anneal

to. Primers also usually contain a restriction site, which allows us to cut open parts of our DNA and paste in what we want. PCR uses a special type of DNA polymerase called Taq, which can work under high temperatures. The first step of a PCR reaction requires denaturation of the DNA causing base pairs to come apart. The primers then anneal to their complementary sequence. The temperature is changed to the optimum temperature of the polymerase is most active to allow for elongation of the sequence. This cycle repeats itself and each cycle causes a doubling in the amount of DNA. Once the DNA was amplified, we tested the PCR samples for proper amplification using DNA gel electrophoresis. We ran a PCR using a forward primer containing the Nhe1 restriction enzyme and a reverse primer containing the Xba1 restriction enzyme.

Once we ran a PCR, we needed to ensure we amplified the correct region of DNA. Sometimes primers may bind each other and form primer dimers or may amplify the wrong regions of DNA. We used DNA gel electrophoresis to do this. DNA gel electrophoresis is a technique to separate DNA fragments according to size. The gel is made of agarose and an electric current is run through the gel to run DNA through the agarose. The top of the gel contains wells to load the DNA and a negative charge. The bottom of the gel contains a positive charge. Since, DNA has an inherent negative charge, the DNA will run from the top (negative) to the bottom (positive) and separate based on size. Larger segments of DNA will not run as far on the gel as smaller segments. Ethidium bromide is added to the gel mix and acts as an intercalating agent that goes in between DNA base pairs and fluoresces under ultraviolet light so that bands can be observed. We noticed bands consistent with the size we expected from our PCR.

We concluded that we amplified the correct DNA segment and were ready to move on to the next step.

The next step involves setting up a restriction digest using restriction enzymes, our target plasmid, and the plasmid containing our gene. Restriction enzymes are enzymes that cut at a specific sequence in the DNA. By cutting our target plasmid and pBOS plasmid using the same restriction enzymes, we created the same sticky ends on both pieces of DNA. This allowed for our gene product that was cut out to anneal to the sticky ends of our target plasmid. We ran the restriction digest on a gel and saw bands. Some bands corresponded to the plasmid reannealing but the band we looked for was that of our gene being inserted into the CamKII plasmid, which has a specific band on our gel.

The next step was to use DNA ligase to connect the ends of the Kirrel3 genes into the plasmid. Annealing involves base pairs forming, but ligation is the linkage of the sugar-phosphate backbone, which is held together by covalent bonds. The DNA ligase enzyme catalyzes this covalent bond formation and allows for the gene to become linked to the rest of the plasmid via a phosphodiester bond between the sugar-phosphate groups on each nucleotide. Once we ligated our gene to our vector, we sent the DNA off for sequencing to ensure that we had the right product from our restriction digest and ligation steps. We also double checked our ligated product by running it on a gel.

Once ligation was complete, the next step was to transform the plasmid in bacteria to grow more of it. Transformation is when bacteria incorporate DNA from the

environment. When we give bacteria exogenous DNA, they can amplify it for us, which is extremely useful as the amount of DNA we obtained from ligation is not enough for further experiments. We transformed DH5alpha bacterial colonies and used ampicillin resistance to select for colonies that incorporated the plasmid. Once this was completed, we grew a colony in liquid broth overnight. After inoculation, the DNA was extracted from the broth through a process called a MiniPrep. In a MiniPrep, we centrifuge the bacterial cells until it forms a pellet. We removed the supernatant to ensure we had cells in our pellet. After this, we lysed the cells open and centrifuged once again to separate portions of the cellular lysate. The DNA is located in the supernatant during this step so we extracted the supernatant and we washed it with buffer and toxin removal solution to purify our DNA sample. Lastly, we eluted the DNA with a centrifugation in distilled water. In order to check the purity of our sample, we checked absorbance on a spectrophotometer. Once the DNA was MiniPrepped, a restriction digest was run to make sure the proper plasmid was isolated. The digest was run on a gel to ensure that the correct size bands appeared and that we indeed had the plasmid we wanted.

The next step in the process is to determine if our cloned plasmid actually expresses in cells. We cultured HEK-293 cells and transfected the plasmid along with known plasmids that worked as a positive control and no transfection as a negative control. To determine expression of a construct, a process known as immunostaining is used. Prior to immunostaining, the cells are fixed in paraformaldehyde to freeze cellular processes and keep subcellular structures in place. The first step of immunostaining

involves using antibodies grown towards a protein (primary antibody) to selectively bind the protein. Antibodies have a high affinity for the protein they bind to and are usually highly specific. Next, a secondary antibody that is tagged to a fluorophore is generated against a primary antibody. This method is used to amplify fluorescence signal when analyzing cells under a microscope. We immunostained our 293 cells and used antibodies raised against HA as well as antibodies against 2A. Antibodies against HA label the epitope tag that is attached to the extracellular domain of Kirrel3. If we see signal from HA, we know that we are also looking at Kirrel3. Antibodies against the 2A protein that allows post-translational cleavage of GFP from 3xHA-Kirrel3.The 293 cells expressed both proteins as expected so we repeated the same experiment using neurons. We also found significant expression in neurons (Figure 4D).

Packaging Viruses

The next step of the process was to package our plasmid into the AAV for brain injections (Figure 2). We cloned an adeno-associated virus using an exosome prep (Hudry et al, 2016). Viruses are prepared by transfecting 3 separate plasmids into HEK-293 cells. One plasmid codes for the viral coat protein. One plasmid is our plasmid of interest that we want to express in mice. One plasmid is a helper plasmid which expresses adenovirus genes and helps the virus come together and become functional. As stated previously, the ITR and WPRE regions of our plasmid assist in the association of this virus in 293 cells. These cells were collected and subjected to multiple rounds of centrifugation. These spins separate the exosomes containing the virus from the rest of the cellular substances. The exosome is a vesicle that contains most of the virus. Thus,

by purifying the exosome, we also have a relatively concentrated AAV preparation. This method works well to create a virus as it is efficient, however there may be some other substances in the exosome as well.

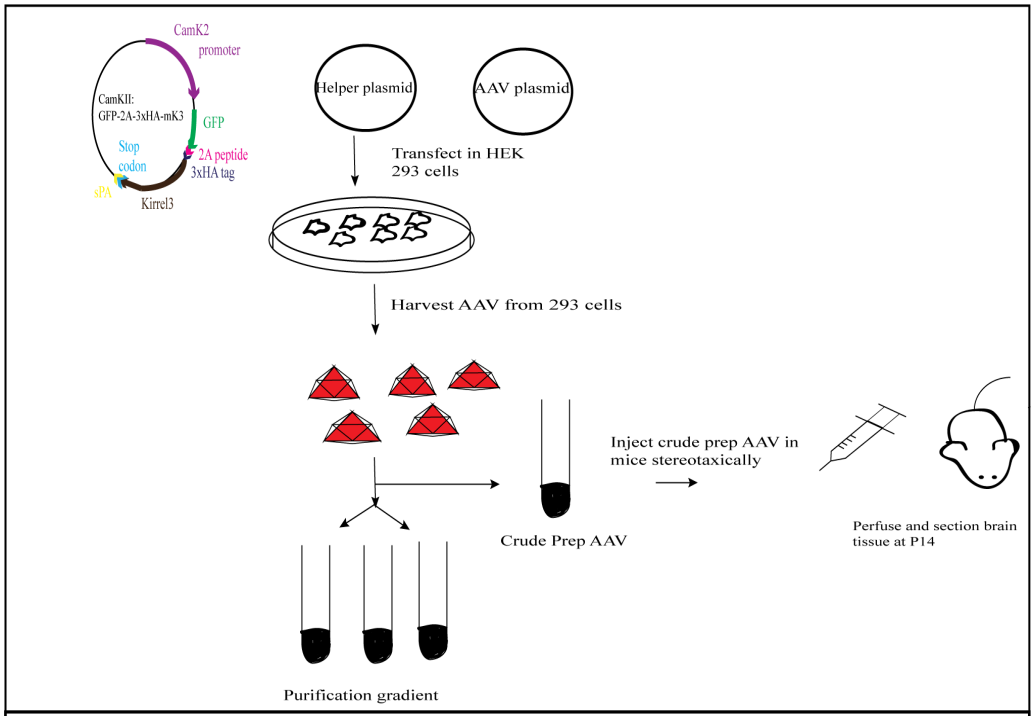


Figure 2. Steps to package an AAV using a GFP-2A-3xHA-K3 construct. Packaged AAVs were tested *in vitro* and *in vivo* for expression.

Testing Viruses *In Vitro*

Once the viruses were packaged with our Kirrel3 gene, we needed to test its infectivity and expression in cultured cells. The Kirrel3 viruses were infected into HEK 293 cells and neurons and immunostained and analyzed for expression. We observed sufficient expression of our virus *in vitro* and were ready to move on the inject the virus into E14 mice (Figure 4C).

Testing Viruses *In Vivo*

We injected pregnant mice *in utero* at postnatal day 14 with our AAV. The pups were born and at day 14, we began to harvest brain tissue. Harvesting brain tissue required a few steps. First, the pups were given various anesthetics and narcotics. Then, we performed a perfusion by injecting phosphate buffered saline (PBS) then paraformaldehyde (PFA) into the circulation of the pup and dissected out the brains. The PBS clears the brain vasculature of blood and other fluids. The PFA fixes the brain tissue. After perfusion of the brain tissue, the brains were sectioned into 50-100 micrometer thick coronal slices using a vibratome. The slices containing the hippocampus were used for further immunostaining. We immunostained the tissue sections and used antibodies for GFP-2A-3xHA-Kirrel3 and our cell filler protein and analyzed under the confocal microscope.

Using this same workflow, we had cloned 3 other constructs in order to determine the AAV that best drives Kirrel3 expression in neurons (Figure 3). Each of the constructs was made using the same techniques and methods and each was packaged into an AAV and tested for expression using the immunostaining and confocal imaging. One construct contained a pMECP2 promoter instead of CamKII, was not packaged in a polycistronic plasmid, and used the FLAG epitope tag rather than HA. This virus was co-transduced with another virus containing GFP. The second construct contained a CAG2 promoter instead of CamKII, was also not packaged in a polycistronic plasmid and also contained FLAG. This virus was also co-transduced with GFP. The third plasmid was a polycistronic construct consisting of mCherry as the cell filler rather than GFP. The construct used FLAG and a CamKII promoter.

# RESULTS

## Development and Testing of Various AAV Constructs

To accomplish detection of Kirrel3 *in vivo*, we needed to design a method of detection for Kirrel3. Currently, there are no antibodies for Kirrel3 that can detect endogenous protein *in situ*, thus we cloned various constructs with different promoters and epitope tags in order to optimize antibody detection and Kirrel3 expression *in vivo*. We then packaged each construct into an AAV and tested the levels of Kirrel3 expression *in vitro* and *in vivo*. Table 1 describes the many constructs we developed and the result of testing *in vivo*. Figure 3 depicts the expression vectors created and the components of each vector. The first construct used the promoter MECP2 (Figure 3A). MECP2 is a promoter that is common in neurons. The advantage of using this promoter is that the sequence was small and thus there was less risk of reaching the packaging limit of 4.7 kb for the virus, perhaps resulting in better expression of our gene of interest. The disadvantage to using an MECP2 promoter is that since it has a small sequence there was a chance it would be weak and not drive strong enough expression of our large packaged gene. Along with the pMECP2 promoter, we included FLAG as the epitope tag and attached it to the extracellular domain of the Kirrel3 gene. For this construct, we cotransduced FLAG-Kirrel3 and a GFP construct to determine localization *in vitro* and *in vivo* (Figure 3A). This construct did not yield effective Kirrel3 expression *in vitro* or *in vivo*.

| Promoter | Virus Prep | Epitope Tag | Result |
|---|---|---|---|
| pMecp2 | Exosome | FLAG | No expression in vitro. |
| CAG2 | Exosome | FLAG | Poor expression in vitro. No detectable signal in vivo |
| CAMKII | Crude | FLAG | Good expression in vitro. Detectable mCherry signal in vivo. No detectable FL-K3 signal in vivo. |
| CAMKII | Crude | 3xHA | Good expression in vitro. Detectable GFP signal in vivo. Detectable 3xHA-K3 signal in cell bodies and dendrites in vivo. |

Table 1. Each of the constructs designed and tested for Kirrel3 expression.

The next construct we used was the similar to the pMECP2 construct except a different promoter was used. The promoter used this time was pCAG2 (Figure 3B), which is larger than pMECP2 and can better drive expression of the gene. CAG2 is a synthetic promoter used to drive high expression of a gene. Furthermore, CAG2 recruits transcription factors and other transcriptional regulators that can drive higher expression of the Kirrel3. The downside to this was that it would push the overall size of our construct close to our packaging limit. This construct also used the FLAG epitope tag. This construct was cotransduced with a GFP construct as well. The CAG2 construct showed low expression *in vitro* and no detectable expression *in vivo*. This is likely due to CAG2's large size causing the construct to approach its packaging limit.

Following this, we decided to create a new type of construct in which our cell filler and our gene of interest would be expressed together in one polycistronic construct. This construct involved the cell filler mCherry, a 2A linker peptide, the FLAG

epitope tag and our Kirrel3 gene (Figure 3C). The advantage of this construct is that any cell visualized by the cell filler will also express the Kirrel3 protein. Furthermore, the promoter was switched out from a CAG2 to a CamKII promoter. The CamKII promoter is the promoter for the calmodulin dependent kinase II, which is a protein commonly found in neurons. Since we used a polycistronic construct that contains a lot of DNA, we decided to use this smaller CamKII promoter so that the construct was less likely to reach the packaging limit of the AAV. Furthermore, the polyA tail from the CamKII construct was smaller, giving us more room to fit the polycistronic construct into our packaged virus.

The last construct we made was similar to the previous construct. Instead of using mCherry as the cell filler, we decided to use GFP, which is well tolerated by cells. From our *in vitro* experiments, mCherry was found to have a lower signal than GFP. GFP, on the other hand, is a well-used cell filler with a well-known antibody that causes increased fluorescence *in vitro* and *in vivo*. We also changed the epitope tag from FLAG to 3 copies of HA in order to potentially increase detection of the epitope tag *in vitro* and *in vivo*. This construct also used the CamKII promoter which was proven to be reliable in the prior construct (Figure 3D).

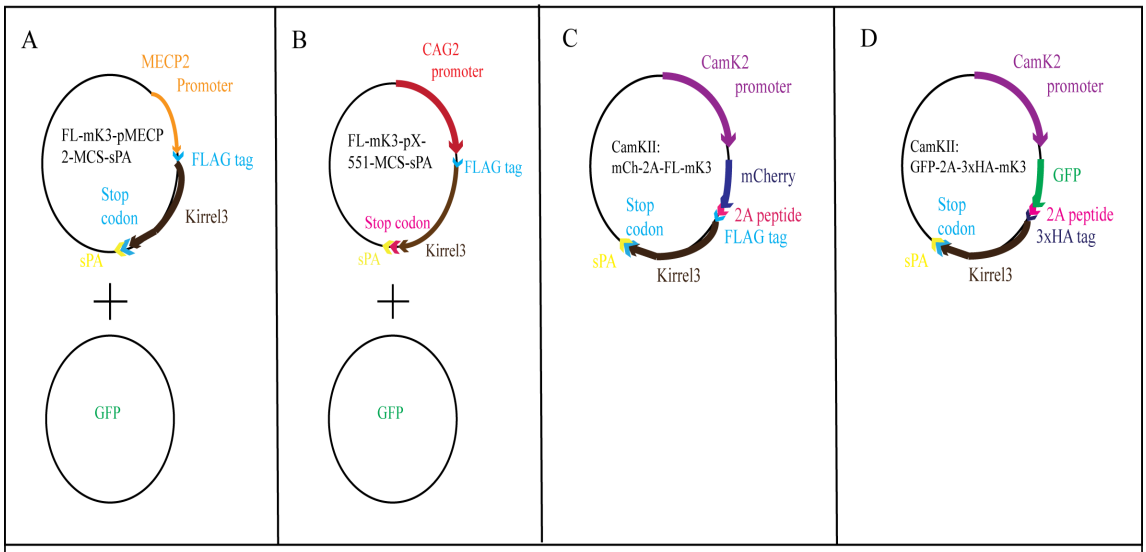


Figure 3. A visualization of the various Kirrel3 constructs. Each construct was packaged in an AAV and tested in 293 cells, cultured neurons and *in vivo* to determine the efficiency of overexpressing the Kirrel3 gene. Cells were examined via confocal microscopy to determine whether or not Kirrel3 expressed.

Expression *In Vitro*

In these set of experiments, the Kirrel3 AAV constructs were used to infect cultured neurons 5 days *in vitro* and were harvested at 14 days *in vitro*, after synapses form in culture. We expected transduction by 14 days *in vitro* to aid in detection of Kirrel3 signal in the cultured neurons. One control for this experiment was transfecting a previously tested and verified Kirrel3 plasmid and a previously tested and verified GFP plasmid into cultured neurons 14 days *in vitro* (Figure 4D). The results from the control experiment showed strong expression of GFP lighting up cell bodies, dendrites, and axons. Kirrel3 expression was also very strong in control experiments as Kirrel3 is detected throughout the cell body, dendritic shafts, dendritic spines, and axons. We used the results of the control as a point of comparison to determine the level of expression of the viral constructs *in vitro*.

*In vitro* testing showed that Kirrel3 is expressed in neurons via AAV transduction but at different levels of expression depending on the construct. The first virus tested, was the virus containing the pMecp2 promoter. Transduction with the AAV:pMECP2-FLAG-Kirrel3 yielded no detectable expression. In contrast, AAV:pMECP2-GFP was easily detected when co-transduced in the same cultures (data not shown). We conclude that this virus did not properly express Kirrel3 at detectable levels *in vitro* and thus is unviable for an experiment *in vivo*. This is likely due to the pMECP2 promoter being too weak to drive expression of the Kirrel3 protein.

The use of the CAG2 virus yielded more positive results. The CAG2 AAV showed weak expression of Kirrel3 *in vitro* (Figure 4A). The signal detected in this experiment was concentrated mostly in the cell bodies of the neurons. Sparse signal of Kirrel3 was found in the dendrites, dendritic spines, and axons, however the signal was too low to draw any conclusions about the localization of the protein. Based on the images, it appeared that the signal found outside the cell body was likely background noise that occurs during immunostaining experiments. This noise was undiscernible from possible true signal from Kirrel3. Interestingly, FLAG-Kirrel3 signal was not detected at higher concentrations of the virus nor at lower concentrations. This finding indicated that FLAG-Kirrel3 expression was highest at an intermediate concentration.

The next construct tested *in vitro* was the polycistronic construct including the mCherry cell filler protein. The advantage of this construct was that we knew that any cell that contained mCherry must have also received the Kirrel3 gene as well. This allowed us to confidently determine that the AAV was packaged and expressed and to

quickly identify transduced cells. In this experiment, we found weak to average expression of Kirrel3 and strong expression of mCherry *in vitro* (Figure 4B). The strongest Kirrel3 signal came from the cell bodies of the neurons. Furthermore, Kirrel3 appeared to localize along the shaft of the dendrites. The Kirrel3 signal appeared stronger than it did for the CAG2 construct, but weaker than the expression in the transfection control experiment.

The last construct we transduced into neurons was the polycistronic construct with GFP as the cell filler molecule and Kirrel3 having the HA epitope tag. This experiment yielded strong expression of both GFP and Kirrel3 (Figure 4C). As compared to the previous constructs tested, this virus showed a definite improvement in detection of both the cell filler and 3xHA-Kirrel3 signal. Kirrel3 appeared to localize along dendritic shafts and some signal was also detected along axons of these neurons. The signal of Kirrel3 was similar to signal from the transfection control experiment and was noticeably stronger than the previous constructs.

Each of the *in vitro* results were interesting and gave insight as to where Kirrel3 would localize in cultured neurons. Using the GFP-2A-3xHA-Kirrel3 virus, Kirrel3 localized to dendritic shafts, areas at which synapses are formed (Figure 4C). Since Kirrel3 is a synaptic cell adhesion molecule, we expected it to localize at the dendritic shafts. Neurons *in vitro*, however, do not build mossy fiber synapses as seen *in vivo* between the DG, CA3 and interneurons. Thus, we needed to perform *in vivo* experiments to determine whether Kirrel3 localizes to these synaptic structures.

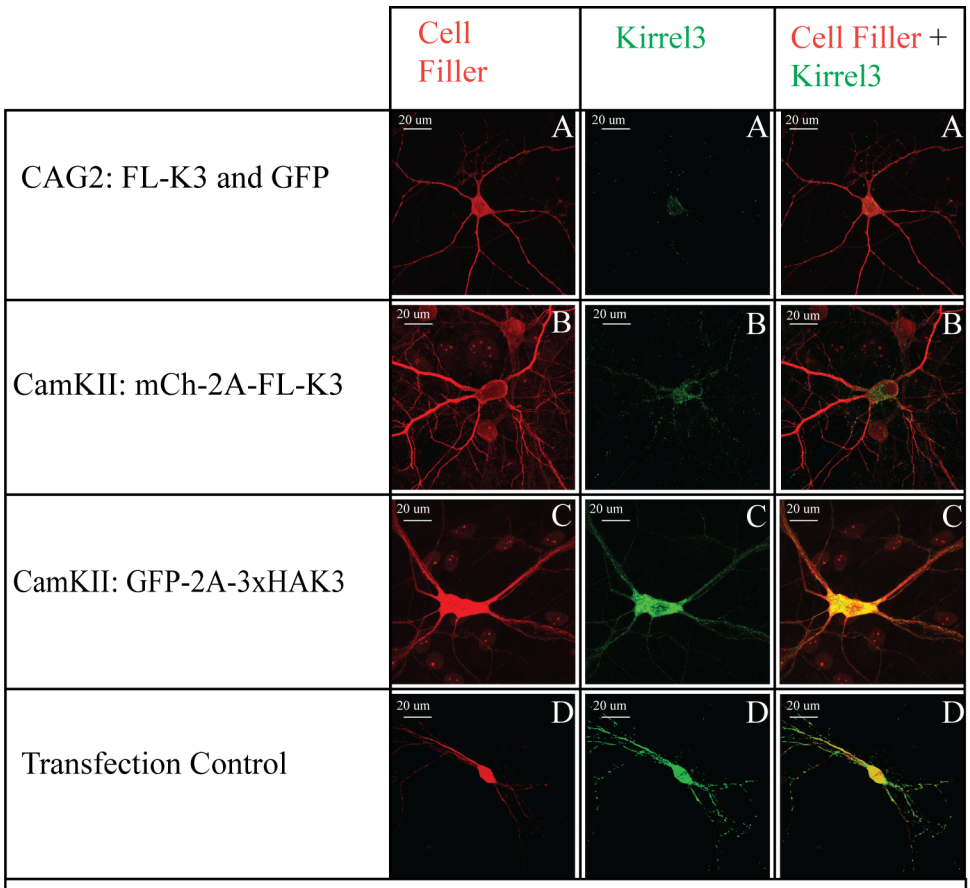


Figure 4. Kirrel3 Localization *In Vitro* Using Various Viral Constructs. A is a construct with a CAG2 promoter cotranduced with a GFP virus and shows low expression of Kirrel3. B is an mCherry construct with Kirrel3 and shows expression of Kirrel3 along the dendritic shafts. C is a construct containing GFP and HA as an epitope tag on Kirrel3. This shows strong expression along dendritic shafts of the neuron. D is a control of a transfected Kirrel3 plasmid cotransfected with GFP and shows strong expression of Kirrel3 along the dendrites.

Expression *In Vivo*

Each of the constructs that showed expression *in vitro* was tested *in vivo* to determine localization. We decided not to test the pMecp2 construct *in vivo* as this construct did not even show expression *in vitro*. Thus, the construct was not used in an *in vivo* experiment. When we tested the CAG2 construct *in vivo*, we injected pups with 2

separate viruses (GFP and FLAG-Kirrel3). After analyzing 50 uM thick sections, we noticed no noticeable FLAG-Kirrel3 signal from this experiment. The GFP virus expressed well indicating that the injections were successful.

Next, we tested the mCherry polycistronic construct. The mCherry signal was strong and expressed in many cells indicating that the virus was efficiently packaged and that the injections were successful. Surprisingly, we did not detect FLAG-Kirrel3 signal above background levels despite its expected polycistronic expression with mCherry (Figure 5B). This is likely due to a combination of post-translation down-regulation of FLAG-Kirrel3 protein levels (compared to the mCherry) and poor detection of the single FLAG-tagged protein using the mouse anti-FLAG antibody in the mouse brain. When cells are overexpressed with the Kirrel3 protein, it is possible that the cell may tag excess Kirrel3 molecules with ubiquitin to tag for degradation to prevent negative effects of having too much of the protein. Furthermore, it is possible that the FLAG epitope induces improper folding with the Kirrel3 protein causing a nonsense folding of the Kirrel3 protein.

Lastly, we tested the GFP-2A-3xHA-K3 construct *in vivo*. The results from this experiment were that GFP expresses well and that 3x-HA-Kirrel3 was found to localize within the cell bodies of DG neurons and along the dendritic shafts of DG neurons (Figure 5A). The appearance of Kirrel3 followed puncta like pattern indicating the presence of synapses (Figure 5C). Kirrel3 was not detected at the mossy fiber boutons nor at mossy fiber filopodia above background levels. This construct showed the best expression of Kirrel3 *in vivo* and was the only construct that allowed us to visualize

Kirrel3 signal in mouse hippocampi. Interestingly, despite strong GFP expression, we only detected 3xHA-Kirrel3 in few cells. Notably, these cells also had the strongest GFP expression.

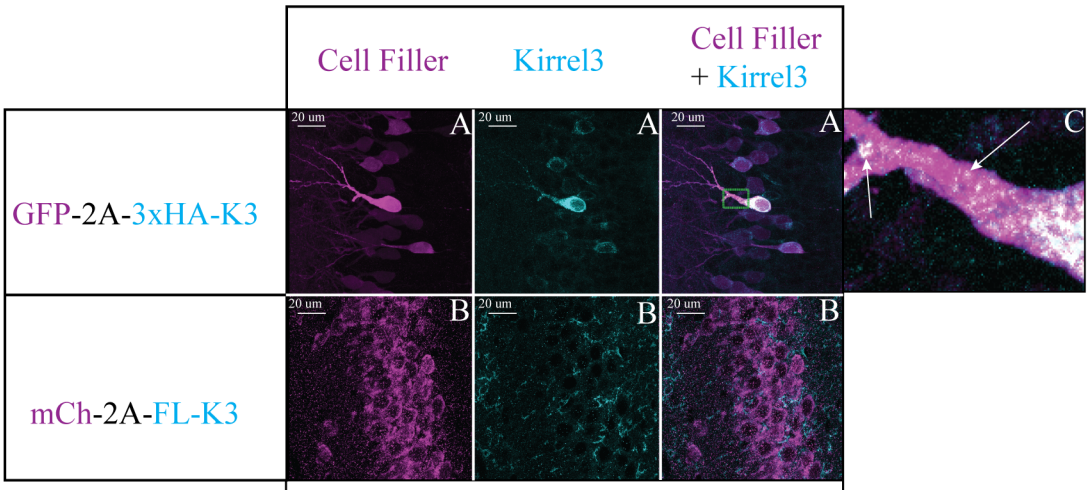


Figure 5. Expression of Kirrel3 *in vivo* using different viruses. A shows the GFP-2A-3xHA-K3 construct expressing Kirrel3 in the cell body and dendrites of the DG neurons. B shows a cluster of CA3 neurons with little to no noticeable expression of Kirrel3. C shows an enlarged view of 5A depicting puncta of Kirrel3 in the DG dendrites.

# DISCUSSION

## Challenges to Studying Kirrel3 Localization

Determining the localization is a challenging prospect and to our knowledge, no previous studies have examined the localization of transmembrane proteins in neurons *in vivo*. Additionally, determining the localization of a lowly expressed transmembrane protein involved in synaptic specificity such as Kirrel3 is even more difficult. The first major challenge in determining Kirrel3 localization is figuring out a way to express Kirrel3 in a mouse. A useful tool in neuroscience, is the AAV, which delivers a gene to the cells *in vivo*. Another possible tool, is to genetically engineer a mouse that has the Kirrel3 gene and a fluorescent marker tagged onto it for visualization with antibodies. A third possibility is using CRISPR-Cas9 technology to knock in Kirrel3 and a fluorescent marker into a mouse's genome and visualize it with antibodies to amplify detectable Kirrel3 signal.

We decided to use an AAV as it is much less costly and provides a method of overexpressing the Kirrel3 gene in a mouse hippocampus. Using an AAV has its challenges and one of the major challenges is the packaging limit of an AAV. AAVs can house 4.7 kilobases of nucleotides before it cannot package anymore DNA. After 4.7 kilobases, the use of an AAV is ineffective and another virus such as a lentivirus may need to be used. It is difficult to balance the size of the construct with the packaging limit. Initially, we co-transduced two separate AAVs; one with FLAG-Kirrel3 and one with GFP, and we could not determine which cells received Kirrel3 and GFP together. Thus, we had to use a polycistronic construct in which we know that if a cell received GFP, it

also received Kirrel3. The issue with the polycistronic construct is that it pushed the size of the gene close to the packaging limit of the AAV which was one reason we picked the smaller sized CamKII promoter. When working with AAVs, this is a challenge to consider and it may be useful to use a lentivirus if working with larger constructs.

Another major challenge with studying Kirrel3 is the lack of suitable antibodies that can tag the Kirrel3 protein. Currently, there are no useful antibodies for Kirrel3 detection, which required us to introduce an epitope tag on the extracellular domain of Kirrel3. This method brings others issues. Signal from epitope tags may be masked *in vivo* due to true protein-protein interactions occurring in the cells. In our experiments, we may have not been seeing HA-Kirrel3 signal in mossy fiber filopodia because the Kirrel3 was interacting with other Kirrel3 molecules to maintain synaptic specificity. Thus, antibodies were not able to bind the epitope tag and give us detectable signal. To determine whether the antibody binds the epitope, we will need to run a western blot of our hippocampal sample. With this technique, the epitope tagged Kirrel3 will be solubilized and no longer be wrapped up in interactions with other Kirrel3 molecules and we can determine whether antibodies are able to bind the epitope or not.

Lastly, when performing any type of overexpression experiment, there is the possibility that overexpressing a protein leads to cellular regulation of that protein. In our experiment, we overexpressed Kirrel3 and it is possible that we could not detect signal due to the cell post translationally down regulating Kirrel3 trafficking to its area of effect. It is important to keep in mind the effects of overexpressing a gene when using this methodology.

Development of Tools for Studying Kirrel3 Localization

The results of the *in vitro* experiments concluded that an AAV is a viable method of introducing the Kirrel3 gene to a population of neurons both *in vitro* and *in vivo*. This was evident from the noticed expression of both cell filler and GFP at comparable levels to the transfection control. Each of the constructs revealed different levels of expression and that may come down to various factors.

The pMecp2 construct that yielded no expression was likely due to the fact that the promoter was small and was likely too weak to drive expression of the FLAG-Kirrel3 gene. Thus, we were not able to detect any expression from this experiment. Furthermore, it was difficult to determine expression in this experiment due to the fact that we co-transduced two different viruses. It is likely that the Kirrel3 virus and the GFP virus hit different cell populations and the only way of determining localization is if a cell was hit by both viruses.

The CAG2 construct worked better than the pMECP2 construct but still did not yield strong expression *in vitro* nor *in vivo*. The CAG2 construct likely had better expression than the pMECP2 because the CAG2 promoter is a larger and stronger promoter for the expression of this gene. The stronger promoter likely led to better expression of Kirrel3 which is why we were able to detect signal in neurons. This signal, however, was still weak in the dendrites compared to the transfection control polycistronic constructs. A possible reason for this is that the CAG2 promoter is large and pushed the virus to its packaging limit. An AAV2 virus can only fit 4.7 kilobases of DNA in it and our construct was 4.6 kilobases. This construct did not provide detectable

levels of FLAG-Kirrel3 *in vivo* as well. For this reason, we could not get conclusive Kirrel3 expression *in vivo* with this construct.

The polycistronic construct containing the mCherry cell filler protein, the 2A linker peptide and FLAG-Kirrel3 yielded strong expression of Kirrel3 in the cell bodies and weak expression of Kirrel3 out in the dendrites. This construct had increased cell body expression than the CAG2 construct and detectable signal in the dendrites *in vitro*. The use of the polycistronic construct was helpful in detection of Kirrel3. We knew that any cells that contained mCherry must have also had Kirrel3. Kirrel3 was detected along dendritic shafts of neurons. This result was consistent with the finding that Kirrel3 is a synaptic cell adhesion molecule. The expression of Kirrel3 in dendrites made this construct a good candidate for *in vivo* expression. We posited that if we were able to detect Kirrel3 signal out in the dendrites of neurons, then this construct would be useful in the detection of Kirrel3 in hippocampal neurons. This construct, however, did not yield favorable results during *in vivo* trials and we had to move on to another construct. The cell filler mCherry showed expression, however, there was no detectable FLAG-Kirrel3 signal above background. Furthermore, the use of the anti-mouse antibody led to signal in the Cy3 channel likely coming from the vasculature of the mouse brain being tagged by fluorescent antibodies. A common aspect in all previous constructs was the epitope tag FLAG. We hypothesized that the FLAG tag may be a reason that we did not detect Kirrel3 *in vitro* or *in vivo*. This epitope may have been tough for the antibodies to bind to and fluoresce from due to Kirrel3's nature of being synaptic. One reason for this could be that the antibody's signal is masked by the interaction of Kirrel3 with its post-

synaptic partner. This interaction may have prevented the antibodies from binding the FLAG tag.

The polycistronic construct containing the GFP filler protein, the 2A linker and 3xHA-Kirrel3 yielded strong expression in the cell body and the dendrites of the neurons *in vitro*. This result showed similar expression levels to the transfection control experiment. The GFP showed more signal than the mCherry which was useful for illuminating the features of the cell, including dendritic spines and axons. Kirrel3 expressed at a higher level and the increased signal is likely due to the switching out of epitope tags. We incorporated 3 copies of the HA epitope tag so we could increase fluorescence from antibody staining. The 3xHA tag overall increased Kirrel3 detection. This viral construct was the most viable of all the constructs as a method of overexpressing the Kirrel3 gene to neurons *in vitro*. The GFP-2A-3xHA-Kirrel3 construct served to solve some of these antibody detection problems. We used the 3xHA epitope tag to try and yield stronger detection of Kirrel3 *in vivo*. The results of this experiment showed expression of Kirrel3 within the cell bodies and dendrites of the DG neurons in the hippocampus. These results are consistent with the previous finding of Kirrel3 mRNA expressing in DG neurons and GABAergic interneurons.

<u>Localization of 3x-HA-Kirrel3</u>

Kirrel3 was found to localize at dendritic shafts *in vitro*. This finding is an indication that Kirrel3 goes to synapses and acts as a cell adhesion molecule. The dendritic shafts *in vitro* have been found to be areas of inhibitory synaptic connections. The finding that Kirrel3 localizes here *in vitro* suggests that Kirrel3 may be functioning at

inhibitory synapses. This finding is consistent with the known expression of Kirrel3 mRNA being expressed in DG neurons and inhibitory interneurons in the hippocampus. It is possible that Kirrel3 plays a role in mediating synapse formation between GABAergic interneurons and DG neurons. In order to determine whether Kirrel3 is truly localizing to inhibitory synapses *in vitro*, we will need to immunostain for a marker that is found at inhibitory synapses such as Vgat, which is found in synaptic vesicles from GABAergic pre-synapses. We can compare Kirrel3 localization to the localization of the inhibitory synaptic marker and determine if Kirrel3 is localizing to these locations *in vitro*.

Another possible explanation for Kirrel3's localization at dendritic shafts is that Kirrel3 is being trafficked along the dendrite to another location. While we may see fluorescence of Kirrel3 in the dendritic shaft, it may be that the protein actually functions elsewhere. One way to properly determine whether Kirrel3 is being trafficked or not is to do a live-labelling experiment in cultured neurons. Live labelling will allow us to track the trafficking of Kirrel3 and determine whether it truly localizes at the dendritic shafts or is just trafficked through the shaft.

*In vivo*, Kirrel3 shows expression in the somas of DG neurons and out into their dendrites in a punctate pattern. Importantly, Kirrel3 is not detected with the mossy fiber boutons of the stratum lucidum layer of the hippocampus. These mossy fiber boutons, are the hypothesized area of localization of Kirrel3, as filopodial development here is regulated by Kirrel3. One possible explanation for this result is that since Kirrel3 is being overexpressed via AAV, that the cells are modifying Kirrel3 in such a way that it

is not trafficked out to the mossy fiber boutons. One possible mechanism for this, is that excess Kirrel3 molecules are being ubiquitinated and degraded. Thus, we are able to detect signal just in the cell bodies and dendrites but not out at the mossy fibers in the axons of the DG neurons.

Another possible explanation for this result is that Kirrel3 truly does not localize to the mossy fiber filopodia and that Kirrel3 functions in the dendrites of DG neurons. This hypothesis, however, would suggest that Kirrel3 has functions that cause downstream effects out into the axons of DG neurons that affect filopodial development and mossy fiber bouton formation. As seen *in vitro*, it is possible that Kirrel3 is acting at synapses between inhibitory neurons and DG neurons since it has been found to localize in the dendritic shafts of DG neurons.

A third explanation for not detecting Kirrel3 is that there is a problem with antibody detection that is not allowing us to detect fluorescence of our epitope tag out in the axons of the DG neurons. One issue could be with antibody penetrance where the antibody is not recognizing and binding the epitope tag. A solution for this is to move the epitope tag to the intracellular domain, which may allow for antibodies to more easily tag the epitope. Furthermore, expressing a similar cell adhesion molecule to Kirrel3 with the same epitope tag will give further insight as to whether there is a problem with epitope tag detection, or whether Kirrel3's signal is decreased due to a biological reason.

Overall, this is a challenging project and it is unclear as to where exactly Kirrel3 localizes *in vivo* and future studies will need to be conducted. We know that Kirrel3 is

being produced in the DG neurons and has puncta in the dendrites of the DG neurons. We also know that an AAV is an effective method of overexpressing the Kirrel3 gene in neurons *in vitro* as well as *in vivo*. Although we are unable to detect Kirrel3 in mossy fiber synapses, we now understand that Kirrel3 is being made in the DG neurons and is possibly being regulated by cells due to overexpression of the gene. We now need to move forward with future studies to determine why Kirrel3 is not being detected at mossy fiber synapses and whether that reason is biological due to overexpression or whether it is due to detection issues with antibodies.

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