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Expression of Channelrhodopsin-2 Localized within the Deep CA1 Hippocampal Sublayer in the Thy1 Line 18 Mouse

Dorothy L Dobbins^{1,*}, David C Klorig¹, Thuy Smith¹, and Dwayne W Godwin¹

¹Department of Neurobiology and Anatomy, Wake Forest University School of Medicine, Winston-Salem, NC, USA

Abstract

Optogenetic proteins are powerful tools for advancing our understanding of neural circuitry. However, the precision of optogenetics is dependent in part on the extent to which expression is limited to cells of interest. The Thy1-ChR2 transgenic mouse is commonly used in optogenetic experiments. Although general expression patterns in these animals have been characterized, a detailed evaluation of cell-type specificity is lacking. This information is critical for interpretation of experimental results using these animals. We characterized ChR2 expression under the Thy1 promoter in line 18 in comparison to known expression profiles of hippocampal cell types using immunohistochemistry in CA1. ChR2 expression did not colocalize with parvalbumin or calbindin expressing interneurons. However, we found ChR2 expression to be localized in the deep sublayer of CA1 in calbindin-negative pyramidal cells. These findings demonstrate the utility of the Thy1-ChR2-YFP mouse to study the activity and functional role of excitatory neurons located in the deep CA1 pyramidal cell layer.

Keywords

Channelrhodopsin; Thy1; Sublayers; Hippocampus; CA1

Introduction

Optogenetic methods have enabled significant advances in neuroscience, particularly in understanding complex circuit interactions (Zhang et al., 2010; Zhao et al., 2011; Kohara et al., 2014), and behavioral functions (Jasnow et al., 2013; Ting and Feng, 2013; Allen et al., 2015). The generation of transgenic animals for optogenetics has provided stable lines with reliable expression patterns between animals (Zhang et al., 2010; Fenno et al., 2011; Ting and Feng, 2013). Further, increased control over the expression of opsins can limit expression to

Corresponding Author: Dorothy Dobbins, Wake Forest University School of Medicine, Neurobiology and Anatomy, 1 Medical Center Blvd, Winston-Salem, 27157, NC, USA, Dldobbin@wakehealth.edu.

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Conflict of Interest

Authors declare no conflict of interest for the work presented in this manuscript.

precise neuronal populations, or cell types, through the use of highly specific promoters (Zhang et al., 2010; Fenno et al., 2011; Zeng and Madisen, 2012; Asrican et al., 2013; Ting and Feng, 2013).

Thy1 was the first of such promoters used to drive expression in transgenic optogenetic animals (Arenkiel et al., 2007), and is still broadly used today (Zhao et al., 2008; Porrero et al., 2010; Fenno et al., 2011; Chen et al., 2012; Asrican et al., 2013; Ting and Feng, 2013). There are a variety of Thy1 transgene founder lines with lines 9 and 18 being the most commonly used (Ting and Feng, 2013). These lines exhibit ChR2 expression in a variety of brain regions, including amygdala, hippocampus, and cortex (Fenno et al., 2011; Asrican et al., 2013; Ting and Feng, 2013). While expression patterns of transgenes are specific and consistent within lines, expression patterns between lines differ. The distribution of Thy 1 transgenes between founder lines has been described in detail by Feng et al. (2000), and others (Arenkiel et al., 2007; Asrican et al., 2013; Ting and Feng, 2013). Transcription of the Thy1 gene dramatically decreases postnatally, contributing to slight variation in expression. This increased variance in expression patterns of Thy1 transgenes among brain regions has been attributed to differential induction of silencing mechanisms, dependent on copy number of transgenes and state of chromatin condensation proximal to their insertion point (Feng et al., 2000).

Thy1-ChR2 line 18, with high expression levels in area CA1, is a particularly useful model for studying hippocampal physiology. Gross histological examination suggests that ChR2 is primarily expressed in CA1 pyramidal neurons (Arenkiel et al., 2007; Asrican et al., 2013). However, there are relatively few studies which have examined cell-type specific expression, and reports in some Thy-1 founder lines indicate expression of ChR2 may be present in interneurons of CA1 (Asrican et al., 2013; Ladas et al., 2015). There are more than 21 subtypes of interneurons in CA1, each playing different functional roles in hippocampal microcircuits (Freund and Buzsaki, 1996; Klausberger and Somogyi, 2008; Bezaire and Soltesz, 2013). In addition, the pyramidal cell population is not a single homogenous cell layer, but rather composed of at least two sublayers that are differentiable based upon physiological features and expression of the calcium buffering protein calbindin (CBN) (Mizuseki et al., 2011; Kohara et al., 2014; Valero et al., 2015). These sublayers receive distinct inputs and are thought to represent different functional pathways within CA1. To identify the specific expression of ChR2 in the hippocampus of the Thy1, line 18 mouse we used cell-type specific markers to perform a histological examination of select interneurons and pyramidal cell sublayers. Our results demonstrate that expression in CA1 is specific to CBN negative, deep pyramidal cells. These findings highlight the utility of Thy1-ChR2-YFP, line 18, mice to generate a better understanding of the role of this specific subset of neurons in hippocampal circuitry.

Methods

Animals

All animals were maintained at Wake Forest University School of Medicine on a 12 hour light/dark cycle. All experiments were approved by the Wake Forest University Animal Care and Use Committee and complied with National Institute of Health guidelines for

minimizing pain and discomfort. Thy1-ChR2-YFP, line 18, mice were obtained from Jackson Laboratory (B6.Cg-Tg(Thy1-COP4/EYFP)18Gfng/J, stock number 007612). Five adult mice, ranging from 8 to 12 months, were sacrificed using Euthasol (Virbac, Fort Worth, TX) and intracardinally perfused with 4% paraformaldehyde. Brains were extracted and stored in phosphate buffered saline (PBS) at 4°C, prior to being sectioned at 50µm with a vibratome.

Immunohistochemistry

Free floating sagittal sections containing dorsal CA1 were chosen randomly across the medial-lateral plane of CA1 for each subject. Sections were incubated in 10mM citric acid at 80 °C for 30 minutes to promote antigen retrieval. Non-specific labeling was blocked by incubating sections with solution of 10% normal donkey serum and 0.4% triton X-100 for four hours. Slices were washed using a 0.4% triton x-100 in PBS mixture. A separate 0.4% triton x-100 in PBS mixture was used to dilute primary antibodies into solution. Sections were then incubated in primary antibody solution at 4°C, overnight. Primary antibodies used included mouse antibody to parvalbumin (1:5000, Swant), calbindin (1:5000, Swant), and rabbit antibody to Wolfram Syndrome 1 (1:250, Protein Tech) (Dong et al., 2009; Kohara et al., 2014). Slices were washed again using 0.4% triton x-100 PBS mixture and then incubated for four hours at 4°C with fluorescence-conjugated secondary antibodies of the same primary vehicle; anti-mouse Alexa 647 (1:500), anti-rabbit Alexa 405(1:500). Sections were rinsed in PBS and mounted to slides. No primary antibody was included in incubations for control sections, and no immunoreactivity was observed in these tissue.

Image Analysis

Hippocampal sections were imaged using Zeiss 710 confocal microscope with a 63× oil immersion lens (NA 1.4). Images were acquired at 1024 × 1024 pixels, with 2× averaging. Single image planes were analyzed for expression of cell markers and ChR2 within the neurons in hippocampal CA1. Two quantification methods were utilized to assess the colocalization of ChR2 with selected cell markers.

First, ImageJ (Schindelin et al., 2015) was used to calculate Mander's overlap coefficient (reported as +/- standard error of the mean, n = number of total slices examined). Given the negative correlation expected between cell markers and ChR2, Mander's coefficient was selected over Pearson's correlation coefficient. Values for Mander's coefficient were averaged across image fields (n = 11–15 per subject) to generate an average overlap value for each cell marker as compared to ChR2 (Dunn et al., 2011).

Second, Spatial correlation was used to calculate absolute number of pixels displaying overlap with ChR2 for each cell marker. These results therefore yield a specific percentage of total shared pixels between the CH2 marker and each tested marker. Using Adobe Photoshop (Adobe Systems, San Jose, CA), thresholding for each channel was performed and remaining binary pixels were assigned by channel to either a red or green respectively. Pixel overlap was determined as regions showing yellow color of green and red overlay and pixel counts recorded assess the total area of colocalization between cell markers. Figures 1F, 2F, and 3F demonstrate absolute expression of markers in red and green, and overlapping

pixels shown in yellow. Spatial correlation is reported as an average of the percentage of colocalized pixels in relation to ChR2 pixels, across the total number of slices examined (n), $\pm$ standard error of the mean. Absolute colocalization was further combined with original merged channel images (Figures 1E, 2E and 3E for better visual display of colocalized regions).

Finally, to maximize visual presentation of ChR2 overlap with the various markers and to further supplement quantitative analyses performed above, intensity histograms were generated. These plot profiles were generated using FIJI for observation of overlap between cell markers as pixel intensity co-varies across a drawn line (Figures 1G, 2G, 3G and 3H). White lines represented in these figures demonstrate the region in which intensity of each cell marker was measured across the distance indicated in the image. Intensity values along this range were plotted against each other for a visual representation and direct comparison of expression for each cell marker.

Results

Expression patterns of Thy1-ChR2 and other Thy1 transgenes have been observed to differ between founder lines. Thy1 transgenes have been found to express in both excitatory and inhibitory cell types, or in certain lines, both cell types (Feng et al., 2000; Allen et al., 2015; Ladas et al., 2015). Thy1-ChR2-YFP line 18 has prominent expression in hippocampus, cortex, and amygdala (Arenkiel et al., 2007; Asrican et al., 2013; Ladas et al., 2015). For a more detailed examination of ChR2 expression within hippocampal CA1 in line 18, sagittal slices were stained with antibodies labeling specific cell types. Sections were incubated with antibody for wolfram syndrome 1, a protein shown to be specific to excitatory cells of CA1 (Takeda et al., 2001; Luuk et al., 2008; Dong et al., 2009; Kohara et al., 2014). Within the pyramidal cell layer ChR2 expression was abundant (Figure 1) and exhibited near complete colocalization with WFS1, ($R=0.913 \pm 0.010$, $n=12$ slices, 5 animals). Average intensity values indicate no difference in intensity levels between the two proteins, verifying ChR2 expression in excitatory pyramidal cells. While there is not absolute colocalization ($\bar{x} = 55.000$, ± 9.678 , $n=12$ slices, 5 animals) between WFS1 and ChR2 expression, this observation may be explained by the gradient of ChR2 expression within the pyramidal cell layer, discussed further below.

There have been conflicting reports of ChR2 expression in interneuron populations in other Thy1 lines (Feng et al., 2000; Asrican et al., 2013; Allen et al., 2015; Ladas et al., 2015). The calcium binding proteins parvalbumin (PV), and to some extent CBN, are established markers of interneurons (Baimbridge et al., 1991; Freund and Buzsaki, 1996; Maccaferri and Lacaille, 2003; Klausberger et al., 2005; Sohal et al., 2009; Kullmann, 2011; Moreno et al., 2012; Donato et al., 2013; Wester and McBain, 2014). While these markers are not representative of all interneurons in the hippocampus, PV expression is observed in the majority of interneurons within hippocampal CA1 (Freund and Buzsaki, 1996; Klausberger and Somogyi, 2008). Given their prevalence and functional role in CA1, expression patterns of each of these calcium binding proteins were examined. In contrast to excitatory cell markers, basket and bistratified interneurons expressing PV did not express ChR2 (Figure 2), ($R=0.200 \pm 0.041$, $n=14$ slices, 5 animals). Examination of average intensity values

support this finding ($\bar{x} = 25.259$, ± 11.887 , $n=14$ slices, 5 animals). In figure 2E–H, ChR2 expression was observed to be decreased in areas with increased PV expression.

While CBN has been shown to be expressed in a subset of interneurons, recent work has highlighted CBN as a marker with the potential to distinguish between distinct sublayers within the hippocampus (superficial and deep) (Sloviter, 1989; Baimbridge et al., 1991; Mizuseki et al., 2011; Valero et al., 2015). Hippocampal slices stained for CBN expression show similar patterns of labeling (Figure 3). CBN positive cells were used as a guide for segregating superficial and deep cell layers when examining sublayer specific ChR2 expression within CA1 pyramidal cells. The areas examined are along the white line in Figure 3F and 3G. ChR2 expression could be seen to exhibit a gradient in Figure 3B and 3D with strong expression in deep pyramidal layer and limited expression in superficial cells. ChR2 expression was not colocalized with CB ($R=0.539 \pm 0.030$, $n=15$ slices, 5 animals) ($\bar{x} = 30.099$, ± 13.159 , $n=15$ slices, 5 animals) and both CBN and ChR2 exhibit differential expression patterns between sublayers. Fluorescent labeling with CBN revealed that ChR2 expression was localized to CBN negative cells of the deep pyramidal layer of CA1.

Discussion

Owing to the precise temporal and spatial regulation made possible by selective expression of opsins, optogenetics affords the ability to study a variety of functions with precise stimulation of neurons. However, different methods for inducing opsin channel expression can generate expression patterns with varying specificity. Our study illustrates the high degree of specificity in expression of ChR2 in the line 18 transgenic Thy1-ChR2-YFP mouse, at least within CA1 of the hippocampus.

Within this region, the complex interaction between excitatory cells and PV basket and bistratified cells, as well as other interneurons, has been well documented (Freund and Buzsaki, 1996; Maccaferri and Lacaille, 2003; Klausberger et al., 2005; Sohal et al., 2009; Bezaire and Soltesz, 2013; Wester and McBain, 2014). Interneurons play a role in the regulation of rhythmic activity, essential to learning, memory and other functions of this brain region (Klausberger et al., 2005; Sohal et al., 2009; Donato et al., 2013; Buzsaki, 2015; Colgin, 2016). Our results indicate ChR2 expression shows distinct expression in excitatory cells with virtually no overlap of opsin channel expression in PV expressing interneurons, similar to the results from Arenkiel et al. (2007) and Asrican et al. (2013). Further, although CA1 of the hippocampus has many interneuron types, and PV expression is not inclusive of all of these, it comprises a large percentage of interneurons in the pyramidal cell layer (Freund and Buzsaki, 1996; Klausberger et al., 2005; Kullmann, 2011; Buzsaki, 2015). This precise expression of ChR2 makes it possible to design optogenetic studies to determine the participation of excitatory CA1 pyramidal cells of CA1 in generating highly synchronous events, and rhythmic patterns of activity intrinsic to this area (Sohal et al., 2009; Klorig and Godwin, 2014; Wester and McBain, 2014; Buzsaki, 2015).

Despite the generalization of pyramidal cells of CA1 as homologous, the existence of distinct sublayers within CA1 were originally described in detail by Lorente de N6 (1934).

Though these sublayers have been known for some time (Sloviter, 1989;Baimbridge et al., 1991) their functional role is only just being explored (Mizuseki et al., 2011;Slomianka et al., 2011;Graves et al., 2012;Kohara et al., 2014;Valero et al., 2015). Deep and superficial sublayers of CA1 can be identified based on distinct gene expression and physiological characteristics (Lorente de Nó, 1934;Dong et al., 2009;Mizuseki et al., 2011;Slomianka et al., 2011;Graves et al., 2012;Valero et al., 2015). The calcium binding protein CBN has been used as a marker of CA1 sublayers, specifically superficial rather than deep (Sloviter, 1989;Baimbridge et al., 1991;Mizuseki et al., 2011;Graves et al., 2012;Moreno et al., 2012;Kohara et al., 2014;Buzsaki, 2015;Valero et al., 2015). Our study confirms the sublayer specificity of CBN to superficial CA1. Further, Chr2 in the Thy1-ChR2-YFP mouse (line 18) exhibits a gradient expression within CA1 cells. Although, not directly discussed similar Chr2 expression was observed in Thy-VChr1, line 8, in the characterization study by Asrican et al. (2013). Our findings indicate greater expression of Chr2 within deep CA1 pyramidal cells that is not colocalized with CBN positive superficial cells.

CBN expression also highlights physiological differences between the two sublayers. Calcium binding proteins allow for cells to have precise regulation over intracellular calcium and further provides protection in the form of buffering from surges in intracellular calcium, which can mediate forms of excitotoxic cell death (Sloviter, 1989;Baimbridge et al., 1991;Slomianka et al., 2011;Moreno et al., 2012). These regulatory and protective roles suggest the lack of these calcium binding proteins may be involved in selective susceptibility to seizure activity (Sloviter, 1989). Here, fluorescent staining with antibodies against these calcium binding proteins indicate Chr2 is not expressed in cells that normally express the calcium binding protein CBN. Should these findings be confirmed through electrophysiology, the specific expression of Thy1-CHR2-YFP, line 18, in CBN negative (i.e., excitatory) pyramidal cells would further highlights the utility of this optogenetic line. Particularly, the ability of these transgenic animals to serve as a model of increased susceptibility to hyperexcitability and seizure activity. Further differences in physiology of these two sublayers have been highlighted in the study by Mizuseki et al. (2011). Deep cells exhibit higher burst activity and firing rates as compared to superficial counterparts, allowing them to participate preferentially in oscillatory activity of this region. A subsequent study performed by Graves et al. (2012) confirmed these physiological characteristics and reported differential responses to neuromodulation of glutamate and acetylcholine between the two cell types.

These differences in functional activity of hippocampal sublayers are proposed to arise from distinct pathways within the hippocampus. For example, the downstream projections of CA1 sublayers have additionally been examined (Mizuseki et al., 2011;Slomianka et al., 2011;Kohara et al., 2014). Deep cells predominantly project ipsilateral, as compared to superficial bilateral projections, and preferentially project to the ventral striatum and nucleus accumbens (Slomianka et al., 2011). These collective physiological responses, combined with a detailed examination of connectivity (Slomianka et al., 2011;Graves et al., 2012;Kohara et al., 2014;Valero et al., 2015) have led to the proposal that deep and superficial CA1 sublayers comprise two streams of information that are functionally cooperative but ultimately, fundamentally segregated (Mizuseki et al., 2011;Slomianka et al., 2011;Graves et al., 2012;Kohara et al., 2014;Valero et al., 2015). The specific localization of

ChR2 in the Thy1 line 18 mouse to deep CA1 pyramidal cells provides the unique ability to more precisely examine the functional role of this sublayer within the hippocampus, and explore its downstream targets. To our knowledge, this is the first example of specific ChR2 expression in deep CBN negative pyramidal cells in CA1.

Our examination of expression patterns of various cell markers in Thy1-ChR2-YFP, line 18, demonstrate this optogenetic line expresses channelrhodopsin in a specific subset of deep excitatory pyramidal cells in CA1. The specific expression of ChR2 in Thy1, line 18 deep pyramidal cells highlights the utility of this line to examine the function of the deep sublayer of CA1 and the specific role of excitatory cells, including a better understanding of distinct synaptic pathways within the hippocampus; their interactions, downstream projections and overall contribution to brain function. Further, given the lack of ChR2 expression in CBN and PV expressing neurons, this line can be used to explore the effects of reduced calcium buffers in neuronal activity and possibly allow for this line to serve as a model of increased hyperexcitability. The unique pattern of expression in this optogenetic line permits more precise interpretation of experimental observations and an overall deeper appreciation of complexity of hippocampal circuitry.

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Highlights

- The Thy1-ChR2 line 18 mice are among the most commonly used for optogenetic experiments and have high expression within the hippocampus.
- Cell specific investigation of this transgenic line using immunohistochemistry reveals, expression of ChR2 is localized to excitatory pyramidal cells of the deep sublayer within hippocampal CA1.
- Given the precise expression of ChR2 within the deep sublayer of CA1, Thy1-ChR2 line 18 has a unique ability to advance the study of hippocampal microcircuitry.

Figure 1.

ChR2 expression in excitatory cells. A. Merged expression of ChR2 and WFS1 through hippocampus at 10× magnification. B. Tile scan of ChR2 expression across CA1 shown in top panel, merged ChR2 and WFS1 expression shown in bottom panel both at 63x. C. ChR2-EYFP expression, D. anti-WFS1 staining, E. Merged image of both WFS1 and ChR2, Mander's Overlap Coefficient, $R=0.913 \pm 0.010$, $n=12$ slices, 5 animals. F. Representation of spatial colocalization thresholding, $\bar{x} = 55.000$, ± 9.678 , $n=12$ slices, 5 animals. G. Regions of intensity measurements, depicted by white line. H. Histogram comparing intensity values for WFS1 (purple) and ChR2 (green). X axes represents position left to right across the measured area in image.

Figure 2.

Comparison of ChR2 expression in PV interneurons. A. Merged expression of ChR2 and PV through hippocampus at 10× magnification. B. Tile scan of ChR2 expression across CA1 shown in top panel, merged ChR2 and PV expression shown in bottom panel both at 63×. C. ChR2-EYFP expression, D. anti-PV staining, E. Merged image of both PV and ChR2, Mander's Overlap Coefficient, $R=0.200 \pm 0.041$, $n=14$ slices, 5 animals. F. Representation of thresholding for spatial colocalization, $\bar{x} = 25.259$, ± 11.887 , $n=14$ slices, 5 animals. G. Intensity measurements were taken at region shown in white. H. Histogram comparing intensity values for PV (Purple) and ChR2 (Green). X axes represents position left to right across the measured area in image.

Figure 3.

Examination of sublayers of pyramidal cells in hippocampal CA1 A. Merged expression of ChR2 and CBN through hippocampus at 10 $\times$ magnification. B. Tile scan of ChR2 expression across CA1 shown in top panel, merged ChR2 and CBN expression shown in bottom panel both at 63 $\times$. C. ChR2-EYFP expression, D. anti-calbindin staining, E. Merged image of both CBN and ChR2, Mander's Overlap Coefficient, $R=0.539 \pm 0.030$, $n=15$ slices, 5 animals. F. Spatial colocalization thresholding, $\bar{x} = 30.099 \pm 13.159$, $n=15$ slices, 5 animals. G and H Merged images of CBN and ChR2 expression, outlining deep (G) and superficial (H) regions of CA1. I and J. Intensity histograms for deep (I) and superficial (J)

expression of CHR2 and CBN, values for CBN (Purple) and ChR2 (Green). X axes represents space left to right across measured area in image.

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