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### Title

DEVELOPMENTAL LATROPHILIN-2 MEDIATED CONTROL OF NEURONAL MORPHOLOGY WITHIN THE ENTORHINAL CORTEX-HIPPOCAMPAL CIRCUIT

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### Author

Liu, Elizabeth D

### Publication Date

2022-02-11

### Data Availability

The data associated with this publication are not available for this reason: N/A

DEVELOPMENTAL LATROPHILIN-2 MEDIATED CONTROL OF NEURONAL  
MORPHOLOGY WITHIN THE ENTORHINAL CORTEX-HIPPOCAMPAL CIRCUIT

By

Elizabeth D Liu

A capstone project submitted for Graduation with University Honors

February 11, 2022

University Honors

University of California, Riverside

APPROVED

Dr. Garret R. Anderson

Department of Molecular, Cell, and Systems Biology

Dr. Richard Cardullo

Howard H Hays Chair and Faculty Director, University Honors

## **Abstract**

Neurons are a group of highly diverse cell types that are assignable to various morphological categories. The most common excitatory neuron type in the brain are known as pyramidal cells. The expression of a synaptic cell-adhesion molecule, Latrophilin-2 (Lphn2), has been previously implicated in the development of synaptic connections in the medial entorhinal cortex (MEC)-hippocampal circuit. To investigate Lphn2's role in neuronal morphologies, reconstructed Lphn2 deficient and normal MEC pyramidal neurons were compared to identify changes in spine type populations across the MEC layers. Using a genetically engineered Lphn2 conditional knock-out mouse model (Lphn2cKO) and performing stereotaxic injections of Cre-recombinase expressing adeno-associated viruses (Cre-AAV), targeted regional specific deletion of Lphn2 expression was achieved. Co-injection of a Cre-dependent tdTomato fluorescent protein expressing virus (DIO-tdTomato-AAV) was performed to visualize tdTomato labeled neurons lacking Lphn2 expression.

In this study, we explore the role of Lphn2 in the regulation of synapses within the MEC-hippocampal circuit by examining compartment-specific spine densities and spine-type changes in defined high Lphn2 expression pyramidal neurons. Changes in spine densities and spine morphology suggests Lphn2 does alter a neuron's normal spine developmental pattern.

## **Acknowledgements**

I would like to take this opportunity to thank my faculty mentor, Dr. Anderson and my graduate student mentor, Jordan Donohue, for all of their continued support and guidance. I would also like to thank my current and former lab colleagues for all of their hard work and input. Lastly, I would like to thank my friends, family, UCR, and the Whitehall Foundation for their support as well.

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## **Introduction**

The brain contains ~82 billion cells called neurons which communicate electrochemically at junctions called synapses. Synaptic connections occur across pre- and post-synaptic neurons, often between pre-synaptic boutons and post-synaptic dendritic spines. These synaptic connections between neurons form the basis of the nervous system and are responsible for receiving and responding to stimuli. Groups of neurons are synaptically interconnected to form neural circuits which are capable of carrying out higher functions, such as learning and memory encoding. The formation of functional neural circuits is a highly specific process requiring the establishment and maturation of precise synaptic connections between established brain regions. Although individual neurons are innately capable of encoding information, the formation of specialized neural circuits allows for more sophisticated information processing.

The proper development of such neural circuits is highly regulated and requires specific timing and molecular combinations. Cell adhesion molecules (CAMs) are trans-synaptic proteins which serve as key molecular players in the initiation and maintenance of these synaptic connections and facilitating synaptic plasticity (Südhof, 2018). These proteins function by guiding and forming adhesive bonds with proteins on neighboring neurons and stabilizing the synaptic connection (Südhof, 2018). These cell-cell adhesion interactions allow for modifications within neural circuits and are crucial in the pruning of extraneous synapses and strengthening of essential spines (Sala et al., 2008). One family of CAM proteins, latrophilins (Lphns1-3), are a family of adhesion G-protein coupled receptors that operate post-synaptically and in tandem with three other families of CAMs (fibronectin leucine-rich transmembrane proteins, neuroligins, and

teneurins) to form intercellular junctions (Anderson et al., 2017). Originally identified as receptors for  $\alpha$ -latrotoxin, a toxin found within black widow venom, latrophilins have since been identified as a molecular contributor to the formation of neuronal networks and synaptic connections (Anderson et al., 2017; Burbach & Meijer, 2019) by mediating synapse specificity (Südhof, 2018).

Interestingly, the deletion of Lphn2 has been shown to change the number of excitatory synapses in the hippocampal and medial entorhinal cortex (MEC) regions (Anderson et al., 2017; Donohue et al., 2021). When examining Lphn2 conditional KO mouse models, it was discovered that Lphn2 deletion significantly reduced dendritic spine populations in the distal tufts of the stratum lacunosum-moleculare brain region and similarly, Lphn2 deletion was also found to affect distal MEC pyramidal neurons in a compartment-specific manner (Anderson et al., 2017; Donohue et al., 2021). Incidentally, Lphn2 is not equally expressed across the layers of the MEC and layers with peak Lphn2 expression displayed altered spine populations (Donohue et al., 2021). These alterations suggest Lphn2 plays an important role in the MEC-hippocampal neural circuit's development and maintenance.

While it has been determined that Lphn2 plays a significant role in the MEC-hippocampal circuit, its effects on specific spine type populations has yet to be determined. Dendritic spines are protrusions from dendrites that make contact with neighboring axons in order to form a synaptic connection (Pchiskaya & Bezprozvanny, 2020). Spines are dynamic and may change their size and shape depending on an organism's neuronal activity and environment; as a result,

they are the likely source of synaptic plasticity (Berry & Nedivi, 2017; Harms & Dunaevsky, 2007). Spine dynamics may be linked to changes in synaptic connectivity and strength, ultimately changing the efficacy of synaptic communication (Harms & Dunaevsky, 2007). Spines are typically classified into four main categories: mushroom, thin, stubby, and filopodia. Each of these four classes have their own distinct morphological characteristics as well as unique contributions to a given neuronal circuit. Each spine type has different contributions to the circuit with mushroom spines serving as mature, large excitatory connections, and thin spines serving as small excitatory connections. Filopodia and stubby spines are classified as immature synaptic connections, but filopodia in particular are highly dynamic and dominate at peak synaptic developmental periods (Berry & Nedivi, 2017). As such, spine morphology analysis is an important method of analyzing the strength of a given excitatory synapse as a correlation has been found between synaptic strength and spine size (Berry & Nedivi, 2017). Features such as spine volume, spine area, and spine length have all been correlated with the effectiveness of synaptic connections and are reflections of function (Al-Absi et al., 2018).

In this study, we dig deeper into the role of *Lphn2* in regulating the synapses within the MEC-hippocampal circuit by examining compartment-specific density and spine-type changes in defined high *Lphn2* expression pyramidal neurons. Neuron morphology analysis can be used to determine if changes have occurred in a given circuit by measuring spine density, size and types as a proxy for synapse development. To identify synaptic strength changes, we compare changes in compartment-specific dendritic spines for different neuronal populations. In particular we examined populations of pyramidal neurons within the distal MEC, a region of the MEC with



high levels of localized Lphn2 expression (Donohue et al., 2021). Using 3-D reconstruction methods, we found a decrease in spine density for secondary apical dendritic compartments, which is accompanied by an increase in spine length and spine density for the apical primary compartment of superficial layer 3 Lphn2 KO neurons (associated with immature spines). This increase is thought to be the result of a compensatory increase in filopodia populations, which are immature spines, to offset decreases in spine densities detected in the apical secondary compartment. This change further suggests Lphn2 alters a neuron's normal spine developmental pattern.

## **Methods**

### **3-D Reconstruction:**

Neuronal image stacks obtained from confocal microscopy were imported into Imaris, a microscopy image analysis software. Identifying information was removed and neurons were subsequently reconstructed by blinded researchers. The soma was constructed using Imaris's "Surface" tool and edited to remove small projections. Imaris's "Filaments" tool was used to manually trace dendrites and axons and Imaris's "Filaments" creation wizard, "Autopath" tool, and "Autocenter" tool were used to automatically recalculate width. Dendritic spines were manually traced using Imaris's "Filaments" tool and Imaris's "Spine Diameter" creation wizard, and "Autopath" tool were used to automatically recalculate spine width.

After neurons were reconstructed, each was broken into eight unique compartments using an overview image: basal primary, basal secondary, apical proximal primary, apical proximal

secondary, apical distal primary, apical distal secondary, tuft intermediate and tuft terminal (as shown in Fig 1).

For each dendritic compartment, spine density was calculated by dividing the total number of spines by the total dendritic length. Data regarding spine max width, volume, and length were automatically measured utilizing Imaris's "Statistics" function. A description of how each measurement was calculated can be found in Fig. 2.

### **Spine Type Classification:**

Spine type classifications were obtained using Imaris's "Spine Classifier" tool. Spine characteristics, outlined by Fig. 3, were used to assign each spine into its respective class based on Imaris's default morphological parameters, Fig. 4.

### **Results and Discussion**

To test if Lphn2 deletion in these defined neuronal populations alters spine density/spine type across all dendritic compartments, mice were injected at P3 using with either (A or B) to achieve sparse neuronal labeling and compare Lphn2 KO with WT neurons. Mice were sacrificed between P50-60 after the circuit is fully mature. Lphn2<sup>fl/fl</sup> mice (Anderson et al., 2017), a transgenic conditional KO mouse line, was utilized to selectively impede Lphn2 protein expression. Control mice were injected with a tdTomato-expressing AAV while KO mice were injected with a Cre-recombinase (hSyn-Cre-AAV5) and Cre-dependent tdTomato AAV (CAG-FLEX-tdTomato-AAV5). Using Imaris, we reconstructed layer 2/3 MEC neurons and analyzed them for changes in spine density and spine class.

## Spine Density

Changes in spine density, defined as the number of spines per  $\mu\text{m}$  of dendrite, offers insight into changes in the number of excitatory synapses and connectivity within a neural circuit. Initial spine density analysis in Lphn2 KO layer 3 superficial neurons revealed an increase in the spine density of the apical primary compartment and a decrease in the apical secondary compartment, consistent with previously reported results (Donohue et al., 2021). This increase is hypothesized to be the result of a compensatory increase in filopodia spines on the apical primary compartment in an attempt to salvage overall synaptic strength. This compensatory increase indicates a lack of Lphn2 protein leads to deviations from normal synaptic development and implicates Lphn2 as being involved in compartment-specific spine development.

In layer 3 deep MEC neurons, both apical primary and secondary compartments showed a decrease in dendritic spine density (Fig. 7&8). This overall decrease in synaptic density implicates Lphn2 as a contributor to the development and maintenance of neural circuitry. As the primary site of excitatory transmissions, a decrease in spine density would suggest a change in the synaptic strength of the circuit.

## Spine Class

While spine density can provide general insight into changes occurring in neural circuits, analysis of differences in spine-type populations can provide specific information about synaptic

strength and circuit remodeling (Berry & Nedivi, 2017). Traditionally, spines are divided into four classes based on their morphological features: mushroom, thin, stubby, and filopodia. Mushroom spines have a large bulbous head and a short neck. Thin spines typically have narrow heads with a width comparable to the spine neck. Stubby spines are categorized as having no necks while filopodia spines are thin, long structures that represent immature synaptic connections and typically lack synaptic function (Berry & Nedivi, 2017; Pchiskaya & Bezprozvanny, 2020).

Preliminary analysis of Lphn2 KO layer 3 superficial neurons showed an increase in spine length in the apical primary compartment (Fig. 5&6). This increase in spine length, when coupled with the aforementioned increase in spine density, appears to indicate an increase in filopodia spines. Such an increase in filopodia population would imply an increase in immature connections and would suggest a lack of Lphn2 may impair the ability to form mature synapses. With no notable differences in other morphological parameters, it is not yet clear whether there are significant differences in the other three spine classes. More in-depth analysis and classification of individual spines may provide specific details on how Lphn2 interacts within the MEC-hippocampal circuit.

Given the change in spine densities and in some morphological parameters, the next step is to classify each spine into a given class. Spine characteristics exist upon a morphological continuum and physical changes to spines represent synaptic changes to the neural circuit (Berry & Nedivi, 2017). Classifying spines will provide invaluable information about Lphn2's effect on

synaptic strength and the maturity of the circuit. Each spine type has different contributions to the circuit with mushroom spines serving as mature, large excitatory connections, thin spines serving as small excitatory connections. Filopodia and stubby spines are classified as immature synaptic connections, but filopodia in particular are highly dynamic and dominate at peak synaptic developmental periods (Berry & Nedivi, 2017). Identifying population differences between control and Lphn2 KO populations will provide information regarding possible circuit changes. Using Imaris's "Spine Classifier" tool, it is possible to assign individual dendritic spines based on morphological parameters described in Fig. 3. Given the aforementioned morphological continuum and variations in experimental conditions (ie: variations in fluorescence levels), changes to Imaris's default parameters are highly anticipated.

## **Conclusion**

Changes in dendritic spine morphology are indicators of remodeling within neural circuits. As such, identifying changes in morphology and spine class populations is one method at measuring changes in circuit remodeling. We observe Lphn2 deletion results in compartment-specific changes in MEC pyramidal neurons. A decrease in spine density in the apical proximal secondary compartment was accompanied by an increase in spine density and length in the apical proximal primary compartment. This increase may be the result of a compensatory increase in the filopodia population, an immature, transient spine class. Filopodia spines are typically present in developing circuits suggesting deletion of Lphn2 may impair proper maturation of neural circuits. While the extent of Lphn2's role in circuit development and maintenance is still

unknown, Lphn2 does appear to play a role in facilitating the right synaptic connections within the entorhinal cortex-hippocampal circuit.

## Figures

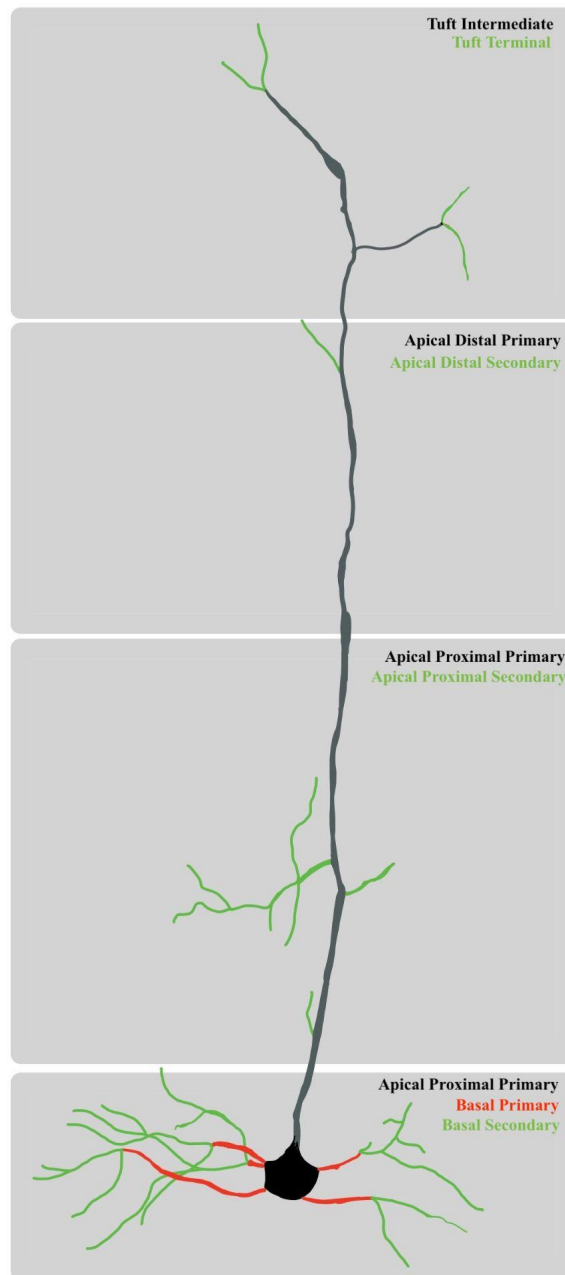


Fig 1. Cartoon rendering of a 3-D pyramidal neuron. The compartments are labelled as the following: basal primary, basal secondary, apical proximal primary, apical proximal secondary, apical distal primary, apical distal secondary, tuft intermediate, and tuft terminal.

Statistical Values	Measurement
Max Width	Maximal value of the spine head diameter
Volume	Calculated by subtracting the hemisphere volume of the spine attachment point from the total volume of the spine frustum and volume of the spine ending hemisphere
Length	Calculated as the sum length of all spine center-line segments including the spine head radius (the tip of the spine) minus the attachment radius (spine part inside dendrite)

Fig 2. Description of how max width, volume, and length values were measured by Imaris “Statistics” function. Obtained from Imaris Reference Manual V 9.2.



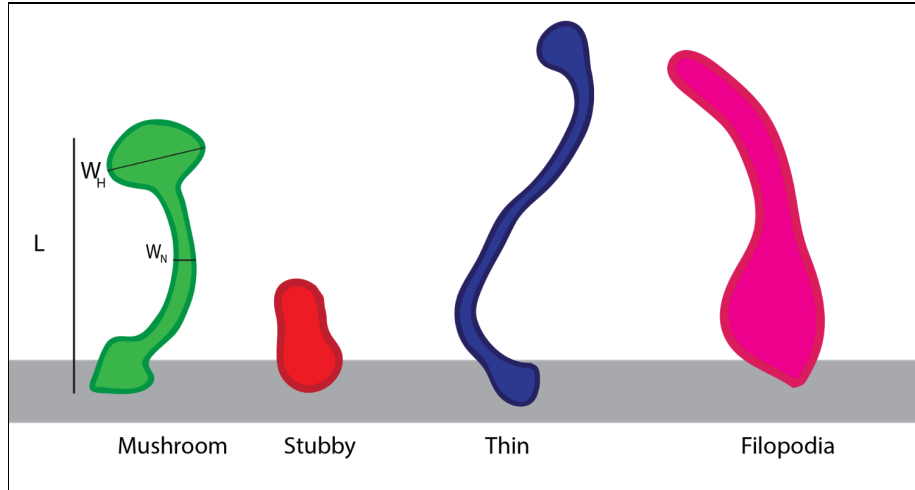


Fig 3. Cartoon rendering of spine characteristics.  $W_N$  represented the maximum width of the spine neck.  $W_H$  corresponds to the maximum width of the spine head.  $L$  corresponded to the length of the spine.

Spine Type	Parameters
Stubby	$\text{length}(\text{spine}) < 1 \mu\text{m}$
Mushroom	$\text{length}(\text{spine}) < 3 \mu\text{m}$ and $\text{max\_width}(\text{head}) > \text{width}(\text{neck}) * 2$
Thin	$\text{mean\_width}(\text{head}) \geq \text{mean\_width}(\text{neck})$
Filopodia	true

Fig 4. Classification of spine morphology using Imaris “Spine Classifier” tool. Parameter rules are applied in order of precedence from stubby to filopodia. Any spines that do not fall within the parameters of stubby, mushroom, or thin are defaultly classified as filopodia.

L3 Superficial					
	Compartment	Spine Density (spines/ $\mu$ m <sup>2</sup> )	Volume ( $\mu$ m <sup>3</sup> )	Width ( $\mu$ m)	Length ( $\mu$ m)
Basal 1°	CONTROL	0.0378	0.0440	0.1178	0.6491
	KO	0.2445	0.1050	0.3350	1.3914
Basal 2°	CONTROL	0.9768	0.1501	0.4340	1.1984
	KO	0.7925	0.1273	0.4033	1.2519
Apical Proximal 1°	CONTROL	0.1420	0.0591	0.1768	0.7061
	KO	0.3565	0.2222	0.3526	1.4898
Apical Proximal 2°	CONTROL	1.9490	0.1500	0.4200	1.2700
	KO	0.9020	0.1800	0.5000	1.1500
Apical Distal 1°	CONTROL	0.6062	0.1162	0.4077	1.3285
	KO	0.5995	0.1756	0.5467	1.5561
Apical Distal 2°	CONTROL	0.9017	0.1708	0.5316	1.2575
	KO	0.3950	0.2100	0.5300	1.2900
Tuft Intermediate	CONTROL	1.3863	0.1565	0.3944	1.3526
	KO	0.5115	0.1756	0.5467	1.5561
Tuft Terminal	CONTROL	0.4690	0.1764	0.4302	1.3274
	KO	0.4690	0.1752	0.5200	1.4250

Fig 5. Average spine density, volume, width, and length values of defined dendritic compartments in MEC layer 3 superficial pyramidal neurons. Data shown is an average from 2 neurons, across 2 independent animals.

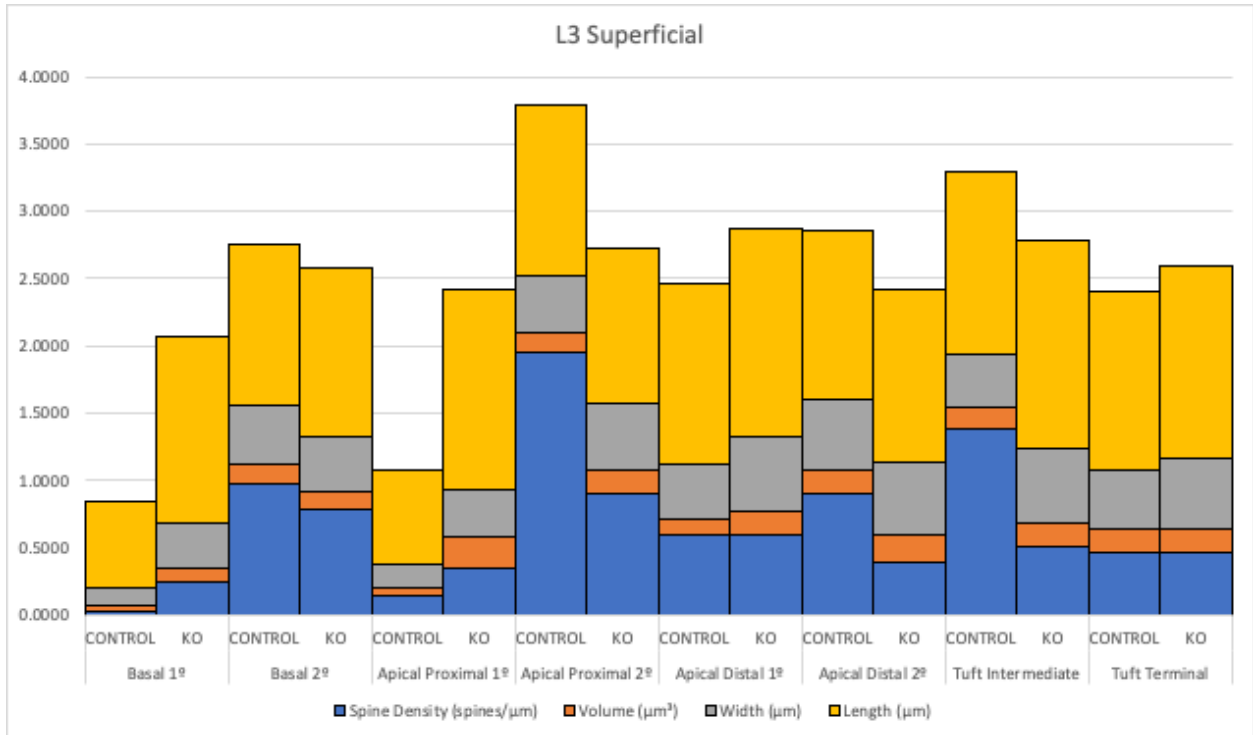


Fig 6. Graphical representation of average spine density, volume, width, and length of defined dendritic compartments in MEC layer 3 superficial pyramidal neurons. Data shown is an average from 2 neurons, across 2 independent animals.

	Compartment ID	Spine Density (spines/ $\mu\text{m}$ )	Volume ( $\mu\text{m}^3$ )	Width ( $\mu\text{m}$ )	Length ( $\mu\text{m}$ )
Basal 1 <sup>o</sup>	CONTROL	0.2635	0.1515	0.2750	1.0429
	KO	0.5735	0.1000	0.4475	1.2788
Basal 2 <sup>o</sup> /3 <sup>o</sup>	CONTROL	0.6213	0.1455	0.4289	1.1900
	KO	0.9243	0.1421	0.4421	1.2936
Apical Proximal 1 <sup>o</sup>	CONTROL	0.4365	0.1401	0.4302	1.3797
	KO	0.4444	0.1781	0.4794	1.4976
Apical Proximal 2 <sup>o</sup>	CONTROL	0.5770	0.1450	0.4100	1.2550
	KO	0.7366	0.1433	0.4310	1.2219
Apical Distal 1 <sup>o</sup>	CONTROL	1.0405	0.1350	0.4000	1.3898
	KO	0.5310	0.0901	0.3842	1.4064
Apical Distal 2 <sup>o</sup> /3 <sup>o</sup>	CONTROL	0.9150	0.2100	0.4500	1.3900
	KO	0.4614	0.0464	0.3021	0.8508
Tuft Intermediate	CONTROL	0.8090	0.1100	0.4230	1.4229
	KO	0.9438	0.1220	0.4169	1.2362
Tuft Terminal	CONTROL	0.5465	0.1535	0.3950	1.2700
	KO	0.6588	0.1403	0.4800	1.5800

Fig. 7. Average spine density, volume, width, and length values of defined dendritic compartments in MEC layer 3 deep pyramidal neurons. Data shown is an average from 3 neurons, across 3 independent animals.

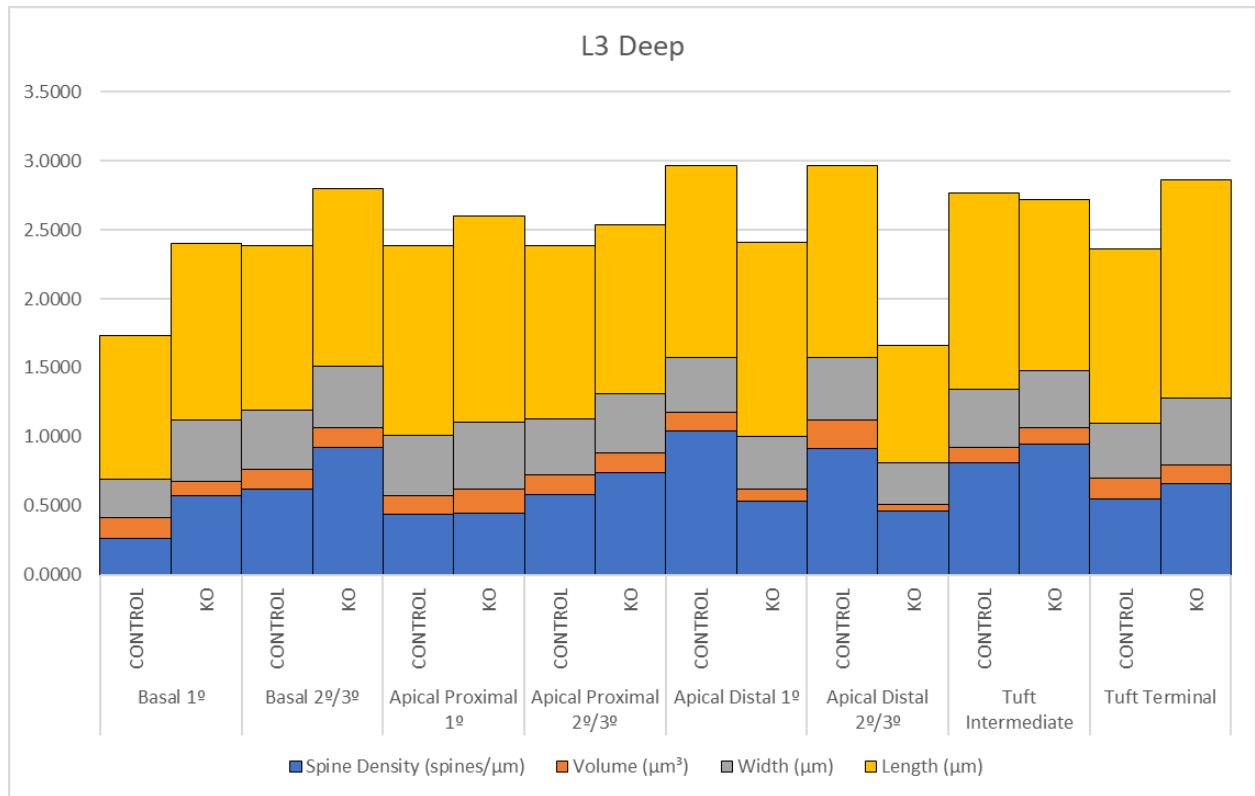


Fig. 8. Graphical representation of average spine density, volume, width, and length of defined dendritic compartments in MEC layer 3 deep pyramidal neurons. Data shown is an average from 3 neurons, across 3 independent animals.

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