Turning On the Off Switch: The Role of Thalamocortically Expressed SHOX2 in Guiding Proper Development of Inhibitory Interneurons in the Barrel Cortex

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Abstract

In the thalamocortical circuit, cortical inhibition is important for fine-tuning the cortical activity of the adult mouse brain. Incomplete or improper development of cortical inhibitory interneurons is associated with disorders such as epilepsy and autism. Cortical interneurons are mainly located throughout the cortical layers of the primary somatosensory cortex (S1). The somatosensory cortex receives direct input from the thalamus indiscrete regions known as "barrels". Each barrel contains a diverse collection of neurons that receive input specific to an individual vibrissa. The ability to visualize the barrel cortex upon immunological staining techniques makes the barrel cortex a helpful tool for investigation of cortical development and plasticity.

In the present study, we will investigate the role of *Shox2*, a transcription factor located in thalamocortical neurons, in cortical development. *Shox2* is known to regulate ion channels important for pacemaking activity in the brain, facilitating well-timed communications between the thalamus and the cortex. By modulating the firing properties and timing of inputs to the cortex, *Shox2* expression may play a role in guiding the development of neurons in S1.

We used viral injections to unilaterally knockout (KO) *Shox2* expression in P6 and P21 mice, and we investigated the role of *Shox2* in postnatal cortical development. Interestingly, unilateral knockout of *Shox2* at P6 resulted in structural abnormalities in the barrel cortex as well as reduced interneuron expression measured at adulthood. Unilateral *Shox2* KO induced after P21 was not found to negatively influence barrel organization; however, *Shox2* KO induced after P21 resulted in interneuron expression abnormalities similar to those seen in P6 KO mice. This research provides supporting evidence for thalamocortically induced maturation of S1 and illuminates the criticality of *Shox2* expression in cortical interneuron maturation.

Introduction

Thalamocortical neurons relay cognitive, sensory, and motor information between regions of the thalamus and cortex and help to integrate sensory information into the neocortex. Development of their cortical connections, otherwise known as axonal projections, occurs during early development and is a helpful model in understanding the circuitry and plasticity of the mammalian brain. Our current understanding of thalamocortical circuitry indicates that projections of thalamocortical neurons to the cortex are necessary for proper barrel formation (Li et al., 2013) and can

affect layer location of inhibitory neurons in the cortex that play a role in determining the proper circuit formation of the cortical system (Zechel et al., 2016). However, the interplay of thalamocortical neurons, their firing properties, and their role in guiding proper circuitry development is an intricate system with numerous novelties yet to be discovered. In this study, we will assess the role of the transcription factor, *Shox2,* in guiding the proper circuitry and development of thalamocortical neurons and their impact on inhibitory interneuron development.

Thalamocortical Organization and the Barrel Cortex

The thalamocortical circuit is composed of excitatory and inhibitory neurons, making the circuit highly specialized for sensory integration. At its most basic, the thalamocortical circuit consists of the thalamus and the cortex. The thalamus receives signals from both the periphery via the brainstem and reciprocal connections via the cortex; the cortex receives and sends information in conjunction with the thalamus and sends signals to other receiving areas of the brain (Zhang & Bruno, 2019). The rodent barrel cortex, which receives information from the whiskers on the mystacial pad of the snout, is used as a model of sensory information processing. From the whiskers to the cortex, the pathway is straightforward. Sensory input from the whiskers first travels towards the brainstem via peripheral and central axons of the trigeminal ganglion primary sensory neurons. After synapsing in the trigeminal nuclei of the brainstem, sensory information travels to the ventral posteromedial nucleus (VPM) of the thalamus. From the thalamus, sensory signals project to the primary sensory cortex where they terminate primarily in layer IV of the cortex (*figure 1*). Sensory information travels along this pathway in parallel nerve tracts corresponding to each vibrissa. These separate tracts are referred to as barrelettes in the brainstem, barreloids in the thalamus, and finally, barrels in the neocortex (Bechara et al., 2015). Each barrel corresponds to a specific whisker and can be visualized through *in vivo* examination of the brain. Cytochrome oxidase (CO) staining, which utilizes diaminobenzidine (DAB) to visualize neuronal activity, is well suited for visualizing the barrel regions of the cortex. In a typical mouse, cytochrome oxidase staining of the flattened cortex will result in numerous darkly stained region in layer IV which each correspond to a single whisker (Woolsey & Van der Loos, 1970).

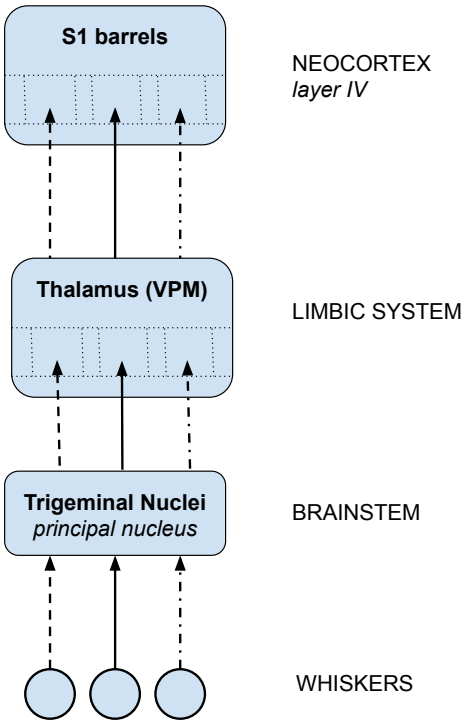

Figure 1. Schematic outline of thalamocortical pathway.

Inhibitory Neurons in the Somatosensory Cortex

The mature cortex contains several types of inhibitory interneurons, all of which play a highly specific role in the cortex. Thalamic neurons project to the somatosensory cortex through glutamatergic excitatory signaling (Hunnicutt et al., 2014). Then, in the somatosensory cortex, thalamocortical signaling is distributed to both excitatory and inhibitory neurons, which then regulate further cortical activity, often by inhibitory mechanisms or feedback pathways (Jones, 1985).

Since previous studies show that thalamocortical projections from the thalamus can affect lamination localization of the inhibitory interneurons in the cortex (Zechel et al., 2016), my study focuses on the main inhibitory target of thalamocortical projections, called parvalbumin expressing inhibitory neurons (Sermet et al., 2019). Parvalbumin expressing neurons modulate signals within the cortex for highly specific control of cortical excitability. For instance, parvalbumin expressing interneurons of the sensory cortex may "shut down" certain stimuli through a feedforward inhibition microcircuit (Tottene et al., 2019). Inhibitory neurons in the cortex are thus an important aspect of fine-tuning excitability in the cortex (Cruikshank et al., 2007). The full mechanism of thalamocortical inhibition is multifaceted and depends on interactions between the thalamus, all layers of the cortex, and many types of inhibitory neurons. However, a fully functioning thalamocortical circuit would be impossible without cortical inhibition. Investigating the input to these inhibitory neurons will teach us how the inhibitory interneurons develop and are regulated.

Development of Thalamocortical Neurons and Cortical Inhibitory Interneurons

Thalamocortical axons organize into separate barreloids and barrels according to their corresponding vibrissa as their axons migrate towards the cortex. Previous studies have shown that glutamatergic input from thalamocortical neuron activity is important for barrel structure (Li et al., 2013). However, since it is still unclear how and when thalamocortical axons organize into these distinct tracts (Senft & Woolsey, 1991), the exact mechanism for thalamocortical driven cortical development is not fully known. There is some evidence that links thalamocortical pathfinding and GABAergic interneuron migration to the cortex. Both thalamocortical neurons and GABAergic neurons have parallel migration timeline in which both enter the cortex at E14 and both wait a few days to begin cortical invasion at E18 (López-Bendito et al., 2008). Other studies suggest that thalamocortical neurons play an important role in the switch of interneurons from tangential migration to radial orientation in the neocortex (Li et al., 2013).

What all of this means is that there is a relative lack of inhibition in the cortex during early development, possibly due to the need to form plastic connection, but there are still inhibitory actors in the cortex before P7. In lieu of parvalbumin cells controlling of the cortex, the younger brain might rely on SST+ inhibitory interneurons to transiently modulate excitatory neurons in layer IV (Marques-Smith et al., 2016). These initial connections appear to be transient, relying on neighboring neurons, and providing only a structure for early postnatal development (Lim et al., 2018). In order for inhibitory neurons to make the transition to parvalbumin expressing neurons, they must receive innervation from the thalamus, which

occurs at roughly P6-P7 (Daw et al., 2007). Dependence on thalamic innervation is an indicator of the integral connection between the thalamic and cortical development.

SHOX2: A Developmental Factor Expressed in Thalamocortical Neurons

Shox2 is a member of the homeobox family of genes containing a 60-amino acid residue motif that represents a DNA binding domain (Blaschke et al., 1998). Significantly, investigation of noncoding elements functioning as enhancers that are conserved between hSHOX and *mShox2* revealed that the deeply conserved sequences are neural enhancers, suggesting ancestral function of SHOX and *mShox2* in the central nervous system (Rosin et al., 2015), justifying investigation of the role of mShox2 in the CNS. The role of Shox2 in nervous system development is unknown, but the Nestin-Cre; $Shox2^{fl/-}$ mouse that causes deletion of *Shox2* in neurons dies at approximately postnatal day (P1) (Rosin et al., 2015), suggesting that CNS-expressed *Shox2* is necessary for survival. The role of *Shox2* varies according to the body region in which it is expressed. However, *Shox2* is best known as a highly expressed developmental factor in both the central nervous system and the heart (Hu et al., 2018), where it serves to regulate pace-making properties. During early development, *Shox2* appears to be an important factor in the proper development of somatosensory neurons in the spinal cord and dorsal root ganglia (Abdo et al., 2011). In the brain, *Shox2* is expressed in neurons of the thalamus which project to the cortex.

Our lab has demonstrated the role of *Shox2* as a transcription factor and its effect on ion channel expression in the mature thalamus (Yu et al., 2021). The mechanism for the pace-making properties of *Shox2* in the thalamocortical circuit is thought to result from modulation of the expression of HCN and Cav3.1 channels, ion channels known to be important for rhythmic firing properties (Yu et al., 2021). In thalamic *Shox2* knockout (KO), reduced expression of HCN type channels is observed. By modulating the expression of these ion channels and their respective currents, *Shox2* plays an integral role in the burst firing of thalamocortical neurons.

Since these channels are critical for the oscillatory burst firing of thalamocortical neurons, *Shox2* KO leads to aberrant thalamocortical circuit activity (Yu et al., 2021). Recent studies from our lab on the effect of *Shox2* KO demonstrate a variety of behavioral abnormalities including sleep disruption, impaired sensorimotor function, and impaired object recognition memory, suggesting a central role in functions associated with the thalamus. It is evident by the current body of research from our lab that *Shox2* plays a critical role in maintaining the function of thalamocortical neurons; however, the role for *Shox2* in the development of thalamocortical projections and their connections are not fully understood and merits further research.

Goals of Experiment and Hypothesis

The goal of this experiment is to study the relationship between *Shox2*-expressing thalamocortical neurons and inhibitory neurons in the somatosensory cortex. Visualizing *Shox2* neurons of the thalamocortical circuit will allow us to better understand the role that *Shox2* takes in maintaining normal function of thalamocortical neurons. We will investigate the role of thalamocortical neurons in guiding the proper development of inhibitory interneurons. Key components of

somatosensory cortex development will be identified and contextualized with relationship to the thalamus.

On a broader scale, understanding the functional development of thalamocortical neurons and their projections will give researchers a glimpse into dynamic modulation of thalamocortical interactions that occur during development and plasticity of the thalamocortical circuit. Proper thalamocortical neuron development is critical for formation of cortical barrels and proper interneuron lamination (Li et al., 2013). Aberrant thalamocortical function has been associated with conditions including epilepsy, autism, and schizophrenia. By understanding the role of *Shox2* in thalamocortical neuron development, researchers will be able to better understand the pathology of such conditions.

MATERIALS AND METHODS

Subjects

All animal procedures were approved by Tulane University Institutional Animal Car and Use Committee (IACUC) according to National Institute of Health (NIH) Guidelines. *Shox2* transgenic mice (*Shox2$^{f/f}$*) mice were generously donated by Dr. Yiping Chen.

Ultrasound and Stereotaxic Injection

To investigate the timeline in which Shox2 may disrupt parvalbumin positive interneuron quantity and barrel formation, two models were created. One model involved the use of a P6 unilateral VB Shox2 knockout mice while the other model involved the use of P21 unilateral VB Shox2 knockout. The benefit of the unilateral injection is that it will allow for a within mouse control in which we can compare the left and right sides of the same mouse.

Our P6 timepoint model was created in collaboration with Dr. Maria Galazo. Dr. Galazo performed unilateral ultrasound injections of a GFPcre virus into the VB of *Shox2$^{f/f}$* mice, creating a within mouse control with *Shox2* KO on the left and control conditions on the right. We allowed these mice to age to adulthood, P35, then euthanize and analyze for disruptions in barrel formation and parvalbumin positive organization in the KO versus CTL sides.

Our P21 timepoint model was created via stereotaxic injection surgeries. We performed unilateral injections of a GFPcre virus into the left VB of *Shox2$^{f/f}$* mice, and control, non- specific RFP virus into right VB, creating a within mouse control with *Shox2* KO on the left and control conditions on the right. We will allow these mice to age to adulthood, P35, then sac and analyze for disruptions in barrel formation and parvalbumin positive organization in the KO versus CTL sides.

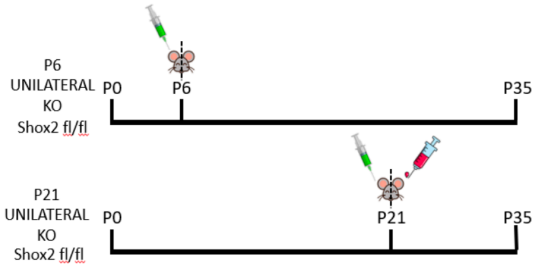

Figure 2: Schematic timeline of experimental timeline in P6 mice and P21 mice. P6 mice received an ultrasound viral injection of GPFcre at postnatal day 6 while P21 mice received a stereotaxic viral injection of GFPcre in one hemisphere and a control virus in the other hemisphere on postnatal

day 21. All mice were matured until P35 and euthanized for analysis.

Tissue Collection

The following section describes the tissue collection process prior to all staining procedures including immunohistochemical staining and cytochrome oxidase staining.
Mice were first transferred from their main holding cage to a temporary cage to prepare for transcardial perfusion. Mice were anesthetized with isoflurane and transcardially perfused with ice-cold PBS followed immediately by 4% paraformaldehyde in PBS until tail rigidity was observed. Mice were then decapitated, and brains were collected for storage overnight at 4 C in a solution of 4% paraformaldehyde in PBS.

Brain for flattened barrel cortex analysis underwent a slightly different preparation. Mice were anesthetized with isoflurane and transcardially perfused with ice-cold PBS followed immediately by 2% paraformaldehyde. Mice were then decapitated, and brains were immediately prepped for flattening. Cortical hemispheres were separated from each other using a razor along the longitudinal fissure. Subcortical structures were then removed from each hemisphere using a razor and a spatula. Excess regions such as the nucleus accumbens, striatum, and orbitofrontal cortex were also removed with a razor to facilitate even flattening. Slices were then placed on a glass slide, cortex down, between two glass capillary tubes. Another glass slide was gently placed on top of the capillary tubes and brain to flatten the slice to the height of the capillary tubes, approximately 1.5mm. The two glass slides were held together with tape and transferred to a container of 1% paraformaldehyde where they would sit overnight at 4 C.

After sitting overnight, brains were sectioned into 50 mm slices using a 0.009" single edge blade attachment on a vibratome (Lancer Vibratome Series 1000). Relevant slices containing either the thalamus or barrel cortex were then suspended in PBS for storage at 4 C until needed for staining.

Immunohistochemistry

Immunohistochemical staining was performed to visualize either PV expressing cell bodies or vGlut2 expressing barrel formations which are both found in the primary somatosensory region (see table 1 for primary and secondary antibodies).

Staining began with washing 50 m slices for three 7-minute cycles in room temperature PBS. Slices were then washed in a blocking solution containing PBS 0.5% TritonX, 3% Bovine Serum Albumin, and 1% Normal Goat Serum for 2 hours before being transferred to microcentrifuge tubes containing the same blocking solution and primary antibodies diluted in a 1:500 ratio. Microcentrifuge tubes were covered in tin foil and stored overnight at 4 C. The following day, slices were washed for three 10-minutes cycles in PBS with 0.5% TritonX. Slices were then washed in the blocking solution containing secondary antibodies in a 1:1000 ratio for two hours. Next, slices were washed for 5 minutes with PBS and 0.5% TritonX to remove excess secondary and blocking solution. Afterwards, slices were washed in a 1:1000 dilution of DAPI for 15 minutes. Finally, slices were washed for 3-minute intervals in one round of PBS and 0.5% TritonX, two rounds of PBS, then one round of PB. Slices were then air dried, mounted on slides using mounting media (Vector

Laboratories, H-1000), and imaged under a confocal microscope.

Type	Host	Antibody	Dilution	Supplier or ID
Primary	Guinea Pig	Anti-VGLUT2	1:500	Synaptic Systems
Primary	Mouse	Anti- Parvalbumin	1:500	Millipore Sigma
Secondary	Goat (Alexa Fluor 488 conjugate)	Anti-mouse	1:1000	AB_2536161
Secondary	Goat (Alexa Fluor 650 conjugate)	Anti-guinea pig	1:1000	AB_2337446

Figure 3: Specifications and concentrations of all immunohistochemically relevant antibodies

Cytochrome Oxidase Staining

Cytochrome oxidase staining was performed to visualize barrels formations in layer IV of the primary somatosensory region. Coronal slices, only, were then analyzed for homogeneity of variance using FIJI ImageJ software.

Slices of interest were first washed for three 5-minute cycles in PBS. Slices were then incubated while rocking in a solution of PBS containing (in mg/ml) 40mg sucrose, 0.5mg Cytochrome C, and 0.5mg diaminobenzidine (DAB). After 3 hours, the reaction was stopped using three 5-minute washes of deionized water. Slices were then air dried, mounted on slides using mounting media (Vector Laboratories, H-1000), and imaged using a compound microscope.

ANALYSIS

Barrel Quantification

Slices that underwent either cytochrome oxidase staining or vGlut2 staining were analyzed using a novel approach which compares the homogeneity of variance using FIJI ImageJ software.

Digital photos of somatosensory region 1 were taken using a Nikon Instrument A1 Confocal Laser Microscope. Images were then imported into FIJI ImageJ software for analysis. First, the barrel region of layer IV was visually identified and manually marked with a digital line approximately 200 pixels in length. The image was then processed using the 'gaussian blur' filter, set to 3.5, in order to eliminate noise. Finally, the predetermined line was analyzed using 'plot profile' which resulted in a visual representation of the pixel intensity over the chosen line.

Data retrieved from 'plot profile' was then exported as a .csv and imported into R Studio. The variance of each data set was calculated, and a paired t-test was implemented to compare the intensity variance the control side to the induced KO side. Significant difference in variation between the two sides would indicate barrel formation disruption.

Cell Counting

Slices stained for parvalbumin were assessed for the quantity of parvalbumin expressing cell bodies.

Digital photos of somatosensory region 1 were taken with Nikon Instruments A1 confocal Laser Microscope. The images were then imported into NIS-Elements C Software where the 'cell count' function was utilized. Three separate sample images were then quantified manually and compared to the digital results. An accuracy of +/- 3 cell bodies was accepted. Cell counts from the control and KO side of the same slice were then compared as a group using the pair t-test.

RESULTS

Parvalbumin Staining and Cell Quantification

Immunohistochemical staining was performed to visualize PV expressing cell bodies in the primary somatosensory region (S1) in P6 and P21 mice (*figure* 4). The side of the cortex that receives input from the Shox2 KO thalamocortical neurons in both P6 and P21 mice had a reduction in the number of visible PV expressing cell bodies. Amongst all samples, there was a significant difference between the number of PV expressing cell bodies on the control side and on the KO side ($T(6) = 4.7263$, $p = 0.003237$) (*figure 5).*

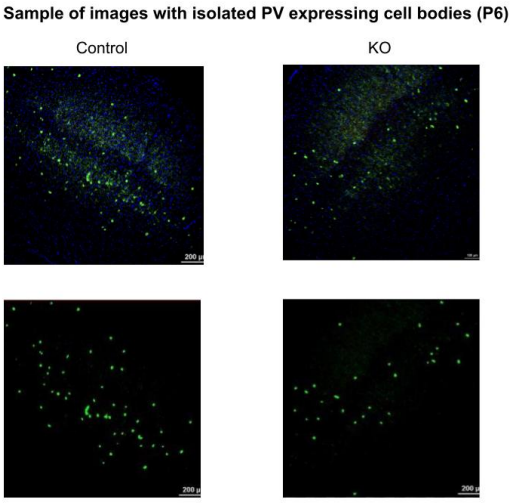

Figure 4. Sample of images used for cell count analysis. Unfiltered image (top) includes blue DAPI as well as green PV+ cell bodies. Filtered image (bottom) includes only PV+ cell bodies isolated in the cortex. The filtered images were used for cell counting.

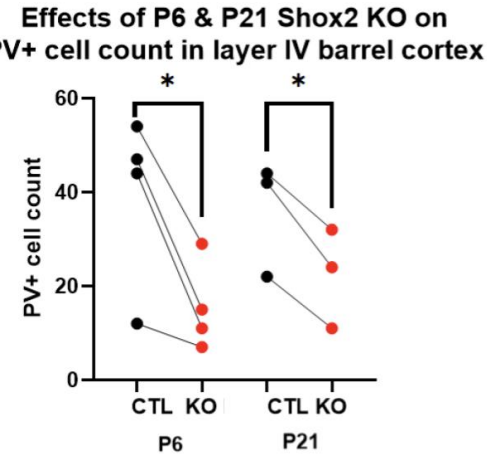

Figure 5: Comparison of PV expressing cell bodies in the primary somatosensory cortex, layer IV, barrel cortex in both P21 and P6 induced unilateral Shox2 KO. In all samples, we observed a greater number of PV expressing cell bodies in the control side vs. the KO side ($T(6) = 4.7263$, $p = 0.003237$).

Cytochrome Oxidase Staining and Barrel Quantification

Barrel morphology of control mice in the study demonstrated normal barrel morphology with separate and discreet regions of darkly stained cells, believed to represent each barrel area. Several dorsal views of the barrels induced at P21 were visually inspected from preliminary analysis of barrel formation and were found to have little morphological differences compared to the side of the cortex which lacked *Shox2* knockout (*figure 6*).

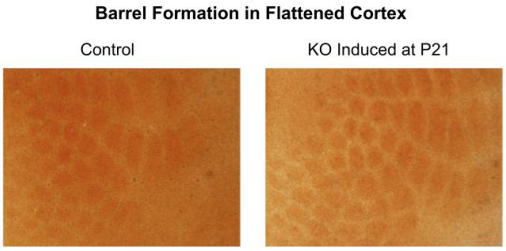

Figure 6: Dorsal view of barrel formation in the primary somatosensory region of unilaterally injected mice. KO was induced at P21 and very little difference in barrel formation was observed.

Both P6 and P21 injected mice with unilaterally induced Shox2 KO were then analyzed for homogeneity of the barrels. Using coronal slices of either P6 or P21 injected mice, the barrels were visualized *(figure 7)*. In each image, only a few of the barrels were visible in a given cross section; however, each slice contained both a control and KO side of the barrels in the same plane. Under the assumption that we should see equal variance in darkness across the barrels, the variance of each side was compared using pixel analysis and a paired t-test (*figure 8)*. Mice with unilaterally induced Shox2 KO at P6 had no significant difference in homogeneity ($T(2) = 3.2003$, $p = 0.08533$). Mice with unilaterally induced Shox2 KO at P21 also had no statistical significance in barrel homogeneity ($T(3) = 2.9061$, $p = 0.06219$) (*figure 9)*.

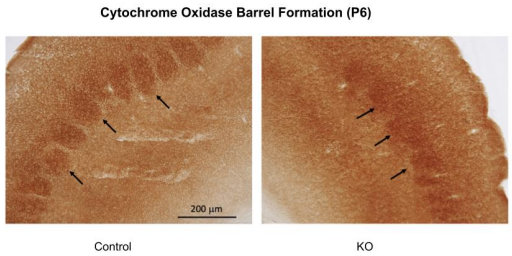

Figure 7: Coronal view of barrel formation in unilateral Shox2 KO induced at P6. Arrows indicate a barrel within the barrel cortex.

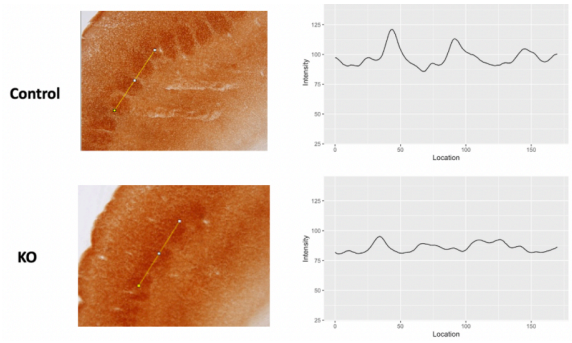

Figure 8. Example of analysis process used to analyze homogeneity in coronal slices with cytochrome oxidase staining. A yellow line represents the region over which color intensity was analyzed. The corresponding graph of the color intensity plot are shown adjacent.

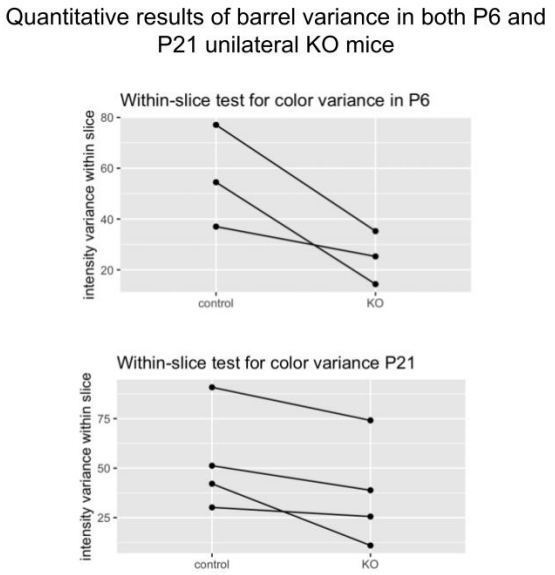

Figure 9: Quantitative results of barrel variance in both P6 and P21 unilaterally induced Shox2 KO. Mice with unilaterally induced Shox2 KO at P6 showed no significant difference in homogeneity ($T(2) = 3.2003$, $p = 0.08533$). Mice with unilaterally induced Shox2 KO at P21 also had no statistical significance in barrel homogeneity ($T(3) = 2.9061$, $p = 0.06219$)

vGLut2 Staining and Barrel Quantification

vGlut2 is an established marker for the barrel cortex in mice aged P3 to adult which is preferentially localized to the axon boutons of thalamocortical neurons, and therefore, indicate barrel areas within the barrel cortex (Liguz-Lecznar & Skangiel-Kramska, 2007). Barrels visualized with vGlut2 immunohistochemical treatment demonstrated similar results to those visualized with cytochrome oxidase. The staining intensity across the barrel cortex had significantly less variance in the KO side of P6 induced Shox2 KO compared to the control side of the same slice ($T(3) = 4.317$, $p = 0.00289$).

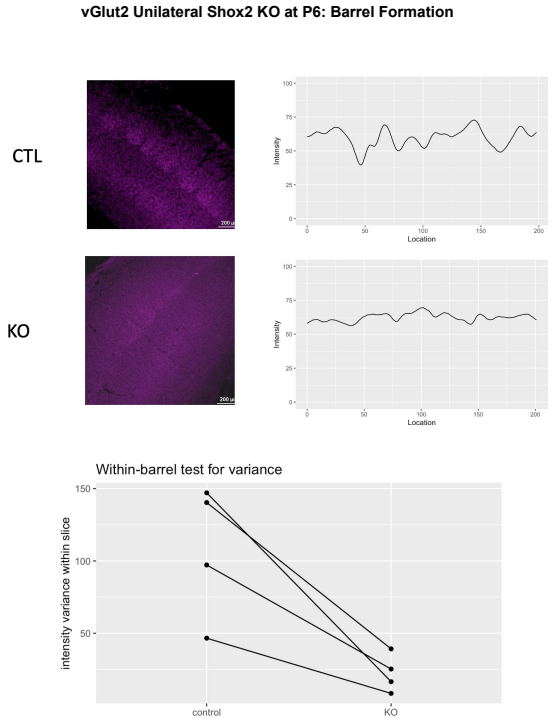

Figure 10: Coronal slices of barrel cortex with vGlut2 staining in P6 induced unilateral Shox2 KO. Sample graphs of the color intensity plot are show adjacent. Below, variance of paired samples in multiple unilateral slices were compared using a paired t-test ($T(3) = 4.317$, $p = 0.00289$).

DISCUSSION

In this study, we found that *Shox2* expressing thalamocortical neurons play a role in the expression of inhibitory neurons in the primary somatosensory barrel cortex. It appears that the expression of these inhibitory neurons in the cortex is dependent, at least in part, on *Shox2* expression in thalamocortical neurons. Interestingly, inhibitory neuron expression was similarly affected by *Shox2* knockout at both day 6 and at day 21 indicating that the maintenance of inhibitory neuron expression is continuously dependent on *Shox2* expression. If this maintenance hypothesis is true, we would

expect future tests on adult mice to also exhibit a reduction in inhibitory neuron expression following *Shox2* knockout. In addition to inhibitory neuron disruption, we found that *Shox2* expression plays a role in the proper development of the barrels in the somatosensory cortex. While data for both P6 and P21 barrel morphology was not significant, these low levels of significance are likely due to small sample sizes and would be improved upon repeated studies. There was, however, a clear trend in the data for P6 knockout mice in which there appears to be a negative impact of *Shox2* knockout in barrel morphology that is not present in the P21 knockout mice. With further supporting research, it might be concluded that *Shox2*-dependent barrel formation is confined to the early stages of barrel development possibly indicating that there is a 'critical window' of barrel development in which *Shox2* expression is necessary. Future studies are needed to confirm that once the barrels have been formed, they no longer rely on *Shox2* expression for their maintenance.

It is still not clear how thalamocortical neurons regulate the inhibitory interneuron expression or barrel cortex maturation, however it is clear that thalamocortical neurons are linked to the development and migration of inhibitory interneurons (Li et al., 2013). Perhaps it is the *Shox2* mediated oscillatory burst firing of thalamocortical neurons which trigger cortical maturation. More likely however, it is not the burst firing alone, but the combination of *Shox2* mediated thalamic input alongside other regulatory processes which cause inhibitory interneurons to fully mature in the barrel cortex. Investigation into the mechanism for which *Shox2* knockout disrupts cortical development could be investigated through the imaging and analysis of thalamocortical projections themselves. Abnormalities of thalamocortical neurons in *Shox2* knockout would illuminate further upstream disfunction in the whisker pathway and perhaps shed light onto the mechanism for *Shox2* mediated cortical disruption.

Future studies should also focus on how these cortical changes brought upon by *Shox2* knockout may affect the physiology of the cortex. It is likely that the downregulation of inhibitory interneurons would result in increases in cortical excitability. Increased cortical excitability has been linked to disorders such as epilepsy and autism and so investigation into the developmental mechanism for cortical excitability could provide insight into the pathophysiology of such disorders.

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