STUDY ON SUB-LETHAL EXECUTIONER CASPASE ACTIVATION IN THE MAMMALIAN NERVOUS SYSTEM

By

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ABSTRACT

During brain development, neurons are formed and create circuits by connecting with other neurons and targets. During this process, excess connections are made and are pruned (removed) to ensure effective functional circuitry. Improper pruning has been linked to developmental disorders such as autism and schizophrenia. Some of these pruning events are coordinated by executioner caspases (EC) in response to pro-apoptotic stimuli. It is well known that executioner caspases have widespread sub-lethal activation in *D. melanogaster* development, however, it remains to be seen whether this is also true in mammalian nervous system development. Previous tools to track EC activation in mammals would only detect a short window of activity and are unable to track cells that have survived executioner caspase activation. We have developed a genetically encoded sensor to detect and map these caspase-dependent pruning events, the *Bmf-iCreER^{T2}* mouse. Once validated, the *Bmf-iCreER^{T2}* mouse will be used to map novel cortical areas undergoing executioner caspase-dependent pruning. This project will contribute to our understanding of circuit refinement events through axonal pruning and neurodevelopmental disorders.

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INTRODUCTION

During brain development neurons are born and form circuits by connecting with other neurons and structures. During this process excess connections are made and these excess connections must be removed to ensure effective and functional circuitry. This process is called pruning. There is a link between improper pruning and developmental disorders, such as autism and schizophrenia (Lawrie et al., 2002 and Thomas et al., 2016). To date, the molecular mechanisms underlying these processes are still not well understood (Hollville & Deshmukh, 2018). However, it is known that some of these pruning events require some of the same machinery involved in the caspase signaling cascade (Cusack et al., 2013).

Caspases are a family of cysteine-aspartate proteases that are directly involved in the pathways that mediate apoptosis and axonal refinement events. In healthy cells, caspases are expressed as inactive zymogens and are activated by proteolytic cleavage, which is a tightly regulated process. Caspases involved in the apoptotic process are subdivided into initiator and executioner caspases (ECs). Initiator caspases (caspases-2, -8, -9, and -10) are responsible for the direct proteolytic activation of ECs (caspases-3, -6, and -7) (Hollville et al., 2018). ECs are a family of effector enzymes that break down proteins and cellular structures for apoptosis, or programmed cell death, in response to pro-apoptotic stimuli.

Apoptosis can be triggered by one of two pathways: intrinsic or extrinsic. The intrinsic, or mitochondrial, pathway begins with the detection of intracellular stress and involves a signaling cascade that leads to Mitochondrial Outer Membrane Permeabilization (MOMP) and the release of mitochondrial proteins, such as Cytochrome c (cyt c) and Second Mitochondria-derived Activator of Caspases (SMAC) from the mitochondrial intermembrane space (Hollville et al., 2018). This pathway is regulated by the pro- and anti-apoptotic members of the B Cell

Lymphoma-2 (BCL-2) protein family, as they are characterized by the presence of BCL-2 Homology (BH) domains. In healthy cells anti-apoptotic proteins restrict Bcl2-associated X protein (Bax) and Bcl-2 homologous antagonist/killer (Bak), the pro-apoptotic proteins, or effectors of apoptosis. Once critical initiators of apoptosis are transcriptionally upregulated, such as *Bmf*, a BH3-only protein, they inhibit the anti-apoptotic BCL-2 proteins, which leads to the activation of Bax and Bak. Upon activation, Bax and Bak form oligomers that cause MOMP with consequent release of mitochondrial proteins, cyt c and SMAC, into the mitochondrial cytoplasm. Cyt c then promotes the assembly of the apoptosome. The Adaptor Apoptotic Protease Activating Factor (APAF-1) oligomerizes upon binding to cyt c and ATP, which promotes the recruitment and activation of caspase-9. The apoptosome will then promote the proteolytic activation of the downstream executioner caspases-3, -6, and -7 (Hollville et al., 2018). These apoptogenic factors promote activation of the caspase cascade, which leads to the cleavage of hundreds of proteins and cell death (Moujalled et al., 2021).

On the other hand, the extrinsic, or death receptor, pathway is initiated when ligands of the tumor necrosis family (TNF) family bind to TNF receptors, which activates caspase-8 and the ECs (Moujalled et al., 2021). This pathway can also connect to and promote the intrinsic pathway by activating a BH3-only protein, such as BH3-interacting domain death agonist (BID), which can lead to an amplified apoptotic cascade (Voss et al., 2020).

Apoptosis leads to the death and degeneration of the entire cell, which includes both the cell body and its axons. Axonal pruning selectively removes excessive axon branches, or dendrites, while maintaining the integrity of the cell body. It is important to note that axonal refinement can occur in both young and mature neurons, and has been reported in a number of regions of the developing and adult nervous system (Geden et al., 2018).

Sub-lethal EC activation has been carefully characterized in *Drosophila* (fruit flies), but has also been discovered in mammals (Ding et al., 2016). During insect metamorphosis, it was found that large-scale pruning allows larval processes to remodel and form adult-specific connections, which requires activation of ECs to occur (Hollville et al., 2018). Furthermore, when ECs are inhibited, axonal pruning is also inhibited. Therefore, it was concluded that ECs are essential for dendrite pruning during metamorphosis, and raised the possibility of the existence of a mammalian-equivalent process during development. ECs are known to be required for large-scale pruning of axons in mammalian retinal ganglion cells (RGCs). During embryonic development, RGCs extend their axons to the superior colliculus, but often extend past their targets. The actions of the ECs eliminate these inappropriate extensions during the first postnatal week in order to refine the eye-specific projection map (Hollville et al., 2018). The fact that this was observed in the mammalian retina suggests that ECs may participate in other refinement events within the mammalian central nervous system.

While both pathways require Bax, caspase-9, and caspase-3, they diverge at the requirements of caspase-6, which is required only for axon-selective pruning, and APAF-1, which is required only for axon degeneration during apoptosis (Cusack et al., 2013). Neurons are known to restrict the apoptotic pathway with maturation by turning off the expression of APAF-1 (Cusack et al., 2013). Neurons may use a non-apoptosome mechanism to activate caspase-9 during axon pruning in order to tightly restrict the direct activation of caspase-3, which would otherwise lead to a widespread apoptotic caspase cascade (Cusack et al., 2013).

Inhibitors of Apoptosis Proteins (IAPs) regulate caspase functions in neurons through direct binding, which may promote their protein degradation. Mammals express different IAPs, but only X-linked Inhibitors of Apoptosis (XIAP) can inhibit caspases-3, -7, and -9 (Ertuck et al.,

2014). Caspase-6 cannot be inhibited by the IAPs (Unsain et al., 2015). In neurons, IAPs keep these caspases inactive to prevent cell death by caspase-3 activation (Ertuck et al., 2014). IAPs are also active during dendrite elimination to keep caspase activity localized in dendrites to protect the soma (Hollville et al., 2018).

IAPs are only active when caspase function needs to be regulated, however, there is not much known about the mechanisms controlling XIAP during local caspase activity. Proteasomal degradation of XIAP has been shown to be required for effector caspase activation during pruning events (Hollville et al., 2018). During apoptosis, IAPs are regulated by their inhibitory proteins. In mammals, SMAC contains an IAP-binding motif (IBM) that antagonizes IAPs (Hollville et al., 2018).

Currently available tools to track EC activation can only detect these events for a short window of time, making it difficult to track and study cells which have previously undergone sub-lethal EC activation. To understand the importance of EC-dependent pruning and its molecular mechanisms we aim to develop a genetically encoded tool to study sub-lethal EC activation in the developing mouse brain. Preliminary data shows that in the mouse retina *Bmf*, a known regulator of caspase activity, is upregulated approximately 100-fold just prior to RGC pruning (data not shown). Using this knowledge, we have developed a genetically encoded sensor to detect and map these sub-lethal EC-dependent pruning events *in vivo*: the *Bmf-iCreER*^{T2} mouse.

We will be using the Cre/loxP recombination system, in which the enzyme Cre recombinase acts on specific DNA sequences called loxP sites. When given tamoxifen, the tamoxifen-activated Cre (CreER^{T2}) will create a fluorescent signal when crossed with one of the many available Cre-reporter mouse lines. Using these genetic tools we will then be able to map

the cells and tissues in the mouse brain that have undergone sub-lethal EC activation. Tamoxifen-dependent Cre recombinase (iCreER^{T2}) will be inserted into the *Bmf* locus, creating the *Bmf-iCreER^{T2}* transgenic mouse line. iCreER^{T2} expression will then be driven by the *Bmf* promoter. *Bmf* is an upstream activator of EC signaling in both pruning and apoptosis that is strongly upregulated during caspase-dependent refinement (Hollville & Deshmukh, 2018). Under exposure to cellular stresses, *Bmf* re-localizes to the mitochondria to promote Bax activation and induce the cleavage of caspase-3 (Akhter et al., 2018).

We will be crossing the *Bmf-iCreER^{T2}* mice with Ai14 mice. The offspring will then express CreER^{T2} under the *Bmf* promoter along with a floxed stop sequence in front of a TdTomato (red) fluorescent reporter at the Rosa26 locus. If the mice are expressing *Bmf* (and therefore CreER^{T2}) at the same time we give them tamoxifen, they will then express the TdTomato fluorescent reporter permanently allowing for the labeling of cells which have undergone sub-lethal EC activation. We will be giving IP injections of tamoxifen at various ages starting with postnatal day 1 (P1) to postnatal day 3 (P3) and looking at retinal ganglion cells in the retina. Pruning in retinal ganglion cells is a well characterized event. It is known that there are pruning events between about P0 to P5 in retinal ganglion cells and in the superior cervical ganglia. This will help us verify that our *Bmf-iCreER^{T2}* mouse is accurately marking pruning events.

The *Bmf-iCreER^{T2}* mouse will be used to identify and map different brain regions and cell types undergoing executioner caspase-dependent pruning. This project will contribute to our understanding of circuit refinement events using sub-lethal EC activation for axonal pruning and its role in neurodevelopmental disorders.

MATERIALS AND METHODS

Animals

Mice were housed in a controlled environment maintained at approximately 22°C on a 12-h light–12-h dark cycle. Mouse chow and water were provided *ad libitum*. All animal procedures presented were performed according to the University of California Riverside's Institutional Animal Care and Use Committee (IACUC) guidelines and approved by UC Riverside IACUC. The *Bmf* mouse line was created using Crispr/Cas9 to favor homologous end-joining by the Gene Manipulation & Genome Editing Core at Boston Children's Hospital. A P2A sequence was inserted after the coding sequence of the *Bmf* gene to minimize disruptions to *Bmf* expression while still maintaining expression of iCreER^{T2} under the *Bmf* promoter. The Ai14 mouse line was purchased from Jax (strain #007914). The C57BL/6N mouse strain was used for RNAscope experiments.

Antibodies and reagents

The RBPMS antibody was purchased from Phosphosolutions (catalog # 1832-R8PMS). The *Bmf* probe was purchased from ACD Bio (catalog # 833011). DsRed antibody was purchased from Takara Bio USA, Inc (catalog # 632496). RNAscope® Assay procedure was performed as previously described by manufacturer, Advanced Cell Diagnostics, Inc with the *Bmf* probe (catalog # 833011) (Wang et al., 2012).

Image acquisition and quantification

Images were acquired by confocal microscopy, using the Leica model DMi8 microscope. *Bmf* puncta were normalized to DAPI area. We quantified the number of average puncta values in the ganglion cell layer by counting the puncta through the analyze particles function in ImageJ.

Shapiro-Wilks test was used to test for normality and a One-way ANOVA followed by Tukey's multiple comparisons test was performed using GraphPad Prism.

BMF-CreER^{T2} genotyping protocol

The genotyping of the *Bmf iCreER^{T2}* mice was performed by PCR using the following primers: forward 5' - GTTCACATGATCAACTGGGCG - 3' and reverse 5' - CTGTACAGATGCTCCATGCCTTT - 3'. The product size is 518 bp.

Tissue Isolation and Processing

Mice were euthanized by CO_2 inhalation and then underwent intracardiac perfusion with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in PBS. All tissues were collected and post-fixed with 4% PFA in PBS at 4°C for 4 hours (IHC) or overnight (RNAscope). Tissues were cryoprotected using 30% sucrose in PBS overnight, then embedded in Optimal Cutting Temperature (OCT) Compound and frozen. All retinal tissue was cut at 15-µm sections using a Leica CM1950 cryostat.

Immunohistochemistry

Add PBST (1x PBS, 0.1% Tween) to remove OCT for 15 minutes. Let dry then draw hydrophobic barrier. 3% Ab blocking buffer (3% BSA and 0.5% Triton X in PBS) in PBS at room temperature for one hour in humidifying chamber. The primary antibodies were then added in antibody buffer (1x PBS, 1% BSA, and 0.5% Triton X) at 4°C in humidifying chamber. The primary antibodies used were Rb α DsRed at 1:500 and GP α RBPMS at 1:250. They were then washed with PBS four times for 10 minutes at room temperature. Secondary antibodies were then added for 2 hours in the humidifying chamber at room temperature. The secondary antibodies used were G α Rb 546 at 1:1000 (Invitrogen, catalog # A-11035), G α GP 488 at

1:1000 (Invitrogen, catalog # A-11073), and DAPI at 1:1000 of 1 mg/mL (Sigma, catalog # D9542). They were washed with PBS four times for 10 minutes. They were then rinsed with ddH_2O and mounted using Fluoro-Gel (EMS, catalog # 7985-10).

Tamoxifen administration

Mice were injected intraperitoneally or orally gavaged at a dose of 150 μg/g tamoxifen (Sigma, catalog # T5648) in corn oil. Young mice (P2-P3) were injected peritoneally for two days then collected 2 days later. P14-P15 mice were orally gavaged for two days and then tissue collected 2 days later.

RESULTS

Between P0 and P14 in this species, the visual system, among others, is under development. Specifically, during ages P0-P6, there is pruning activity in the retinal ganglion cell layer (Simon et al., 2012). To address whether *Bmf* would be a suitable driver for iCreER^{T2}, we studied the retinal ganglion cell layer using RNAscope (a type of fluorescent *in situ*) during and after these pruning events. *Bmf* transcripts appeared as puncta under a fluorescent microscope and are quantifiable. We examined the retinal ganglion cell layer at different postnatal ages: P0, P3, and P14. As expected, the expression of *Bmf* RNA is increased during pruning events (P0-P6) (Figure 1A). However, after pruning activity has subsided (P14), *Bmf* RNA expression decreases (Figure 1B).

Even though our data set is a bit limited and thus underpowered, the data presented trended towards significance between P0 and P14 (P=0.0548, Figure 1B). The graph shows the mean ± SEM for all ages, where n=4. The measured values are: 0.75 ± 0.4 puncta/μm^2, 0.46 ± 0.2 puncta/μm^2, and 0.29 ± 0.1 puncta/μm^2 for P0, P3, and P14, respectively. Therefore, the average puncta values varied for the different postnatal ages with an overall decrease in *Bmf* RNA expression after pruning events.

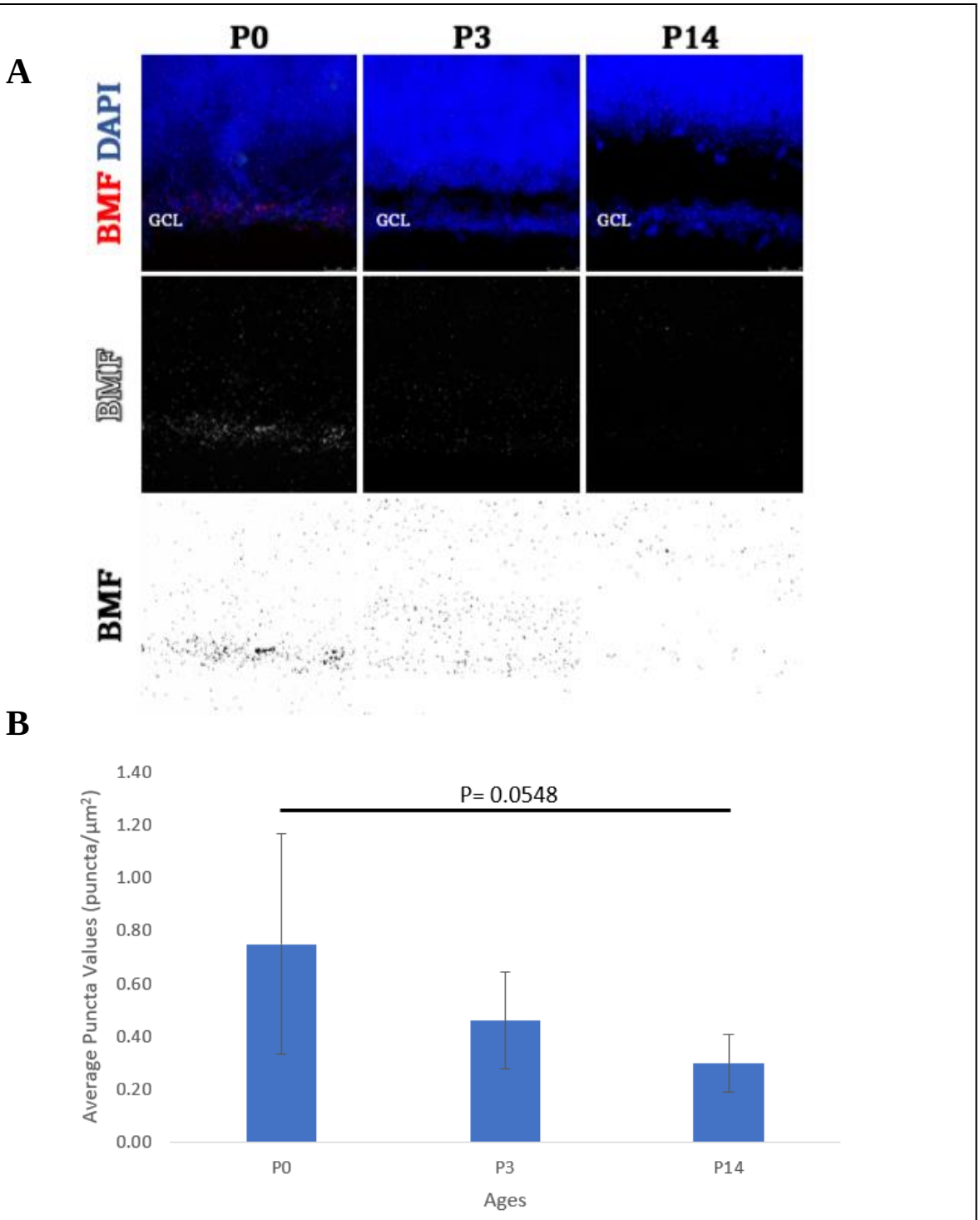

Figure 1 | Expression analysis of *Bmf* in the retina. (**A**) Quantification of the *Bmf* puncta within the retinal ganglion cell layer (ImageJ) is shown above. *Bmf* RNA is labeled via *in situ hybridization*. Expression of *Bmf* increases during a known pruning event. After pruning activity, *Bmf* expression decreases. (**B**) The average puncta values varied for the different ages: P0, P3, and P14. Data represents the mean ± SEM (n=4). The P-value was calculated using one-way ANOVA followed by Tukey's multiple comparisons test. Scale bar, 25 µm.

Based on this and other more conclusive observations suggesting enhanced expression of *Bmf* during pruning (Figure 1; data not shown), we generated a tamoxifen-dependent Cre knock-in into the *Bmf* locus (*Bmf-iCreER*^{T2}). When crossed to the Ai14 reporter line, if the mice are expressing *Bmf* (and therefore CreER^{T2}) at the same time we give them tamoxifen, they will then express the TdTomato fluorescent reporter, permanently allowing for the labeling of cells which have undergone sub-lethal EC activation. The mice at ages P2-P3 were injected peritoneally with tamoxifen for two days then collected at P5. The P14-P15 mice were orally gavaged with tamoxifen for two days and then tissue collected at P17.

Qualitative observations from our recordings confirmed that at ages P2-P3, TdTomato is expressed when tamoxifen is given. At P15-P16, no TdTomato is seen, even with the presence of tamoxifen. Although further quantification for these images is not presented, Figure 2 suggests that the *Bmf-iCreER*^{T2} mouse is capable of labeling cells that have experienced sublethal EC activation and can be utilized in the future to label EC-dependent pruning events in the brain.

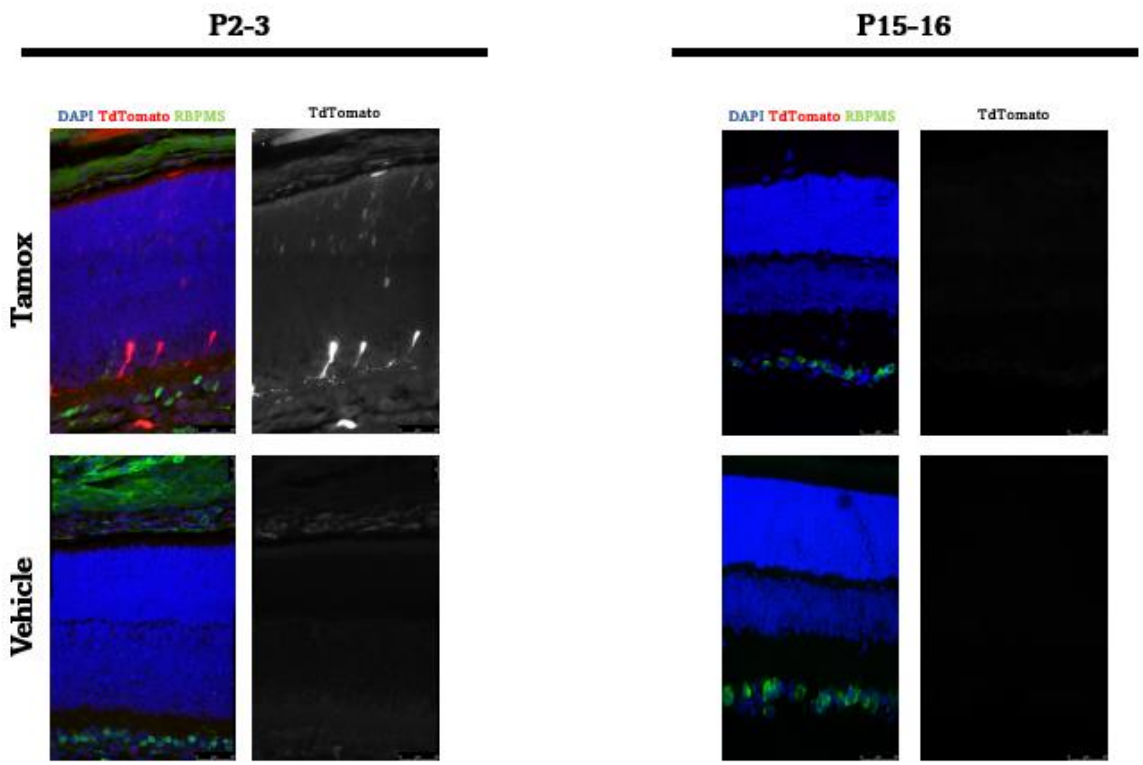

Figure 2 | *Bmf-iCreER^{T2}* labels RGCs during pruning. Images of P2-3 and P15-16 ganglion cell layers with Tamoxifen and oil are shown above. At ages P2-3, TdTomato (red) is expressed in when tamoxifen is present. At P15-16, TdTomato is no longer expressed. Scale bars, 25 μm.

DISCUSSION

In this study, we have investigated whether the *Bmf-iCreER^{T2}* mouse is accurately marking pruning events by studying the retinal ganglion cell layer. Using our *Bmf-iCreER^{T2}* mouse, we have studied whether cells that have undergone sub-lethal EC activation were labeled, providing a unique opportunity to study the areas in which there is executioner caspase-dependent pruning.

Previously, there have been studies focused on locations within neurons that are involved in axonal refinement events as well as the mechanisms that limit them to that area only. For example, in Low et al, they examined if certain proteins were involved in the regulation of stereotyped pruning of motor corticospinal tract axons by injecting a fluorescent retrograde tracer into the superior colliculus to examine the distribution of the motor neurons that extend axon collaterals to the superior colliculus (Low et al., 2008). Compared to this study, they observed these effects at later postnatal ages, such as P25. In Williams et al, they found that caspase activity is only confined to the dendritic compartment of pruning neurons and is not detected in the soma or axon (Williams et al., 2006). This study aims to create a shift towards locating other EC pruning events within the mammalian nervous system.

In Cheng et al, they used a Calbindin2-EGFP (CB2-GFP) transgenic mouse where GFP is selectively expressed by OFF-αRGCs to allow for the visualization of OFF-αRGC axons in retinorecipient targets across development and into adulthood (Cheng et al., 2010). In Hong et al, they aimed to visualize RGC axon arbors by using transgenic mice that express a tamoxifen-activated Cre recombinase (CreER) in BD-RGCs, which are ON-OFF direction-selective cells, to label and reconstruct individual RGC arbors over development and in response to sensory deprivation (Hong et al., 2014). These studies address only part of the questions we are hoping to

address by either aiming to understand the mechanisms involved in localized caspase activity or using transgenic mice to visualize targets within the retinal ganglion cell layer. However, together we will someday find a solution to visualize pruning events in novel cortical areas within the mammalian nervous system through our studies.

Some limitations within this work includes a small sample size (n=4), which skews the data as it has low statistical power and increases the margin of error.

Further studies are suggested in other areas where sub-lethal EC pruning is prominent, such as the superior cervical ganglia or the superior colliculus. One could also look into later postnatal dates (P14 and above) to see which cells survive caspase activation and to identify any new changes produced.

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