Rapid pruning of thalamocortical axons during monocular deprivation in mouse primary visual cortex

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Summary

A single paragraph of fewer than 150 words. The primary goal of the Summary should be to make the general significance and conceptual advance of the work clearly accessible to a broad readership. References should not be cited in the Summary.

Running title (<50 words): *Rapid axonal plasticity during visual deprivation*

Highlights

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Introduction

Long-term monocular deprivation (MD) in cats and primates causes a dramatic structural rearrangement of thalamocortical (TC) axons in the primary visual cortex (V1). While this famous example of cortical structural plasticity mirrors the functional shift in ocular dominance (OD) of visual cortical neurons, TC axon structural plasticity lags behind the physiological plasticity of neuronal responses (Hubel and Wiesel, 1998).

[[SPINE HERE?]] Consequently, it remains controversial whether MD-induced anatomical rearrangements of TC axons in V1 serves as a mediator of rapid functional plasticity or is simply a long-term consequence of rapid physiological plasticity (LeVay et al., 1980; Antonini and Stryker, 1993). Addressing this controversy would address a key unanswered question in the field: Are synapses that undergo LTD-like changes during MD subject to disconnection and elimination in the visual cortex?

In cat V1, significant reductions in the terminal arbor complexity of TC axons subserving the deprived are only detectable days after the functional OD shift, an observation that has reinforced a passive role for structural plasticity (Antonini and Stryker, 1993). Similar studies in mouse V1 provided no evidence of TC axonal branch retractions or other anatomical indicators of weakened input after 20-40 days of MD, thereby providing further support for the view that structural changes to TC circuitry are not required for physiological OD shifts to manifest (Antonini et al., 1999). In contrast however, results from recent studies show that structural changes indicative of input weakening occur in TC synaptic input to layer 4 of V1 and actually parallel the OD shift in mice (Coleman et al., 2010), which is characterized by a rapid (1-3 days of MD) depression of deprived-eye responses (Frenkel and Bear, 2004; Liu et al., 2008) and a weakening of TC transmission (Khibnik et al., 2010) in layer 4. [[Turn to layer 1 and spines here? More recently, it has become possible to…]]

However, the fact remains that large-scale changes to TC axons that lie somewhere between reductions/shrinkage in synaptic terminal and terminal arbor size and that are temporally matched to functional response depression have not been directly observed (Feldman, 2009). Thus, the idea that structural changes to TC input act in concert with synaptic and physiological plasticity to contribute to the earliest functional consequences of MD remains controversial (Espinosa and Stryker, 2012). To address these outstanding issues, we used eye-specific labeling of TC axons and chronic two-photon laser-scanning microscopy to image these axons in mice during MD. Using this approach, we were able to directly test the hypothesis that

retractions of deprived-eye TC axonal branches are rapid enough to contribute to MD-induced deprived eye response depression in V1.

Experimental Procedures

Animals and surgery

Male C57/BL6 P15-17 mouse pups were received from Charles River and housed under 12 h light:12 h dark conditions at MIT. All procedures were performed in accordance with NIH guidelines for humane handling of animals and were approved by the Institutional Animal Care and Use Committee at MIT. Monocular eyelid sutures and intraocular injections were performed as previously described (Coleman et al., 2010). Only mice whose eyelids were completely fused were included in the study.

Cranial window surgeries were performed on the left hemispheres of P18 mice as previously described (Strobl et al., 2016) and as follows. Prior to the craniotomy, a small headpost was affixed to the skull with glue and bone cement anterior to bregma and used to secure the head in a modified stereotaxic frame. The injection site was measured 1.70 mm posterior from bregma and 3.30 mm lateral from the midline suture. Injections were made at an angle in the left hemisphere (the head was rotated 30 degrees clockwise). Injections were made by slow infusion (14 nl pulses every 15 s over 2 min) at 2.60 mm and 2.30 mm below the dural surface. The pipet was left in place for 5-10 min prior to slow withdrawal. A custom-made 4.0-mm glass coverslip (No. 1 thickness, Thermo Fisher Scientific) was placed directly on the dura and anchored to dry skull using high-viscosity cyanoacrylate glue (Loctite #454). Glue was applied all around the coverslip to create an airtight seal. Bone cement (Palacos-R) was then applied over the glue and to the skull immediately surrounding the window. Any remaining exposed skull was then covered with dental cement (Orthojet) and the subjects were allowed to recover under a heat lamp after receiving subcutaneous injections of antibiotic (Baytril, 8 mg/kg) and Lactated Ringer's solution (0.2-0.4 cc). Subjects were individually housed and maintained on an oral suspension of sulfamethoxazole (1 mg/ml) and trimethoprim (0.2 mg/ml) in their drinking water.

Lentiviral vectors

Lentiviral vectors were prepared as previously described (Coleman et al., 2003; Strobl et al.). Virus was suspended in 100 µl of Dulbecco's PBS with calcium and magnesium.

Optical intrinsic signal imaging

Functional mapping of the binocular region of V1 was achieved using optical imaging of intrinsic signal as described previously (Kalatsky and Stryker, 2003). Subjects were imaged 1-2 weeks post surgery with the exception of 4 subjects (2 control mice, 2 MD mice), which were imaged after the two-photon imaging sessions. Briefly, subjects were lightly anesthetized with 0.5-1.0% isoflurane. Images of cortex lying under the cranial window (from ~600 µm below dura) and illuminated with red light (610 ± 10 nm) were continuously obtained using an imaging system (Imager 3000/C system, Optical Imaging Inc.). The visual stimulus consisted of a thin white horizontal bar (w x h - 73deg x 2deg) on a gray background drifting at a temporal frequency of 1 cycle/12 s and was presented for 60 cycles on an LCD monitor. For data analysis, images were spatially binned by 4 x 4 pixels and the cortical intrinsic signal was obtained by extracting the Fourier component of light-reflectance changes that matched the stimulation frequency ($12\ s^{-1}$). The response magnitudes in these maps are fractional changes in reflectance and a threshold of 30% of peak response amplitude was applied to the response magnitude map to define the border of each region. The entirety of V1 was determined by binocular visual stimulation and binocular V1 borders were delineated by ipsilateral-eye stimulation.

Two-photon imaging

Following a 2-3 week recovery period, imaging of TC axons was performed using a Prairie Technologies Ultima two-photon laser-scanning microscope fitted with a Ti:Sapphire light source (Chameleon, Coherent). Axons labeled with mGFP were imaged at a wavelength of 930 nm. Z-stacks were acquired with a step-size of 1.0 µm using an Olympus 20X water-immersion lens (N.A. 0.95). Laser power was incrementally increased by the acquisition software with increasing cortical depth and ranged from 20-60 mW at the objective. Two baseline images of 3-8 different regions were acquired (2.5X zoom; 512 x 512 pixels; pixel scale = 0.45 x 0.45 µm). Image sessions typically lasted 1-2 h depending upon the number of regions. Mice were randomly assigned to a group and the imager was blind to group after baseline imaging.

Two-photon image analysis

Branch tips. Branch tip measurements were performed as described (Strobl et al., 2016). Two baseline images were acquired prior to MD (and subjective) MD. Only imaged regions and structures that could be readily identified in the 2 baseline (pre-MD) images were used for

further analysis. All 3-D tracing and analyses were performed blind to condition using Neurolucida (MicroBrightField, Inc.) or the 'simple neurite tracer' plug-in for Fiji/ImageJ image processing software (http://fiji.sc/wiki/). All analyses were performed blind to condition using custom-written software for MATLAB (MathWorks). In some cases, a second, independent blind researcher confirmed branch tip identification and measurements. The measurement noise (σ_{noise} = 1.38 µm) for images was computed as previously described from (De Paola et al., 2006). The net change in branch length (nΔL) was calculated as the average change in length of all branches from each group relative to a previous imaging day for each imaging interval as follows: $n\Delta L_{MD0} = L_{day(0)} - L_{day(-3)}$, $n\Delta L_{MD3} = L_{day(3)} - L_{day(0)}$, and $n\Delta L_{MD7} = L_{day(7)} - L_{day(0)}$ where day indicates the imaging day relative to MD (or subjective MD for controls). Branch tips were further classified as retracting ($\Delta L < 0$) or extending ($\Delta L > 0$) post-MD by calculating ΔL as follows: $\Delta L_{pre} = L_{day(0)} - L_{day(-3)}$, $\Delta L_{MD3} = L_{day(3)} - L_{day(0)}$, and $\Delta L_{MD7} = L_{day(7)} - L_{day(3)}$. Since branch tip length changes $\geq 3\sigma_{noise}$ (<4.14 µm) reliably indicated actual branch movement (change in length) outside of the measurement noise, retractions or extensions were operationally defined as those with a root-mean-square displacement $\geq 3\sigma_{noise}$, which were used to calculate the magnitude of length changes.

En passant boutons (EPBs). EPBs were classified and tracked along spared axonal branch regions. First, segmentation of each Z-stack was performed using the "fill out" function of Fiji-simple neurite tracer (with "threshold" setting of 0.3-0.4). Putative bouton regions, defined as bright swellings along the axon backbone, were marked in maximum-intensity Z-projections of segmented axon arbor tracings and stored using the region of interest (ROI) manager tool in Fiji. Putative gains were selected by identifying bright swellings on post-baseline images. The brightness of each bouton ROI (~2.5 µm x 2.5 µm) was then calculated by summing the pixel gray values. Next, the average brightness of the axon backbone was calculated using measurements acquired from 5-µm wide segments along the backbone, where at least one background ROI was typically located within 7 µm of each bouton ROI. Relative brightness was calculated by dividing the bouton ROI intensity by the average brightness of the 2 nearest backbone ROIs. Putative boutons were then classified using custom-written MATLAB software that designated each bouton ROI as a true bouton if its total brightness was >1.5X the average backbone brightness on at least 1 baseline imaging day and >1.3X the average backbone brightness on the other baseline imaging day. A loss was scored as one that fell below 1.1X the average backbone brightness on subsequent imaging days. A gain was scored as a bright

swelling identified in a region of the axon that was not scored on baseline images, but was >1.3X the average backbone brightness on subsequent imaging days. Analogous criteria have been previously shown to predict actual synapses with high fidelity (De Paola et al., 2006; Grillo et al., 2013). EPBs from each subject were pooled and used to calculate the percentage of dynamic EPBs, EPB losses (L) and EPB gains (G): as follows: %lost = 100*(nL/nEPBs); %gained = 100*(nG/nEPBs).

Histology

For all cases, the location of lentivirus injections and labeled dLGN cells relative to the contra- and ipsilateral eyes was confirmed using confocal microscopy. Subjects were perfused and 100-µm thick serial sections were cut on a vibratome as previously described (Coleman et al., 2009). Only subjects that had clear labeling in dLGN and whose injections had avoided labeling the dorsal portion of lateral posterior nucleus, which also projects to binocular V1, were included for axon analysis. For a subset of subjects used in this study, the eyes ipsilateral to the imaged hemisphere were injected with CTB-tagged with an Alexa-fluor dye (555 or 647) 4-5 days prior to perfusion and used for histological study (see below) (Fig. 1).

In addition to functional imaging of intrinsic signals, in some cases the location of imaged areas in V1 was confirmed by labeling experiments as previously described. Briefly, a small hole was drilled in the glass coverslip using a diamond bit (1/12" bit, 3/32" shank; DiamondBurs.Net, LLC) fitted to a high-speed micro drill. Approximately 100 nl of CTB-555 was injected 450 µm below the dural surface of the imaged area, which was identified by surface vasculature. CTB-647 was then injected into the eye ipsilateral to the cranial window. Histological analyses was used to confirm that CTB-555 was located in binocular V1 and that retrogradely labeled cell bodies located in the medial portion of dLGN and overlapped with the ipsilateral-eye projection zone.

Confocal imaging and lentiviral vector injection analysis

Confocal images were acquired for analysis as previously described (Coleman et al., 2009). For the dLGN images, all sections with identifiable GFP-labeled neuronal soma were imaged for post-hoc confirmation of lentiviral vector injection location and a subset of brains were used for quantitative analysis as follows. The mGFP reporter was localized to neuronal membranes, which was optimal for axon labeling, but made it difficult to clearly delineate labeled neuronal somas. Therefore, high-magnification image Z-stacks consisting of 15-20 0.2

μm-thick optical sections (pixel size = 0.3 μm x 0.3 μm) were carefully examined and locations (XY coordinates) marked with square ROIs, which were saved using the ROI manager in Fiji/ImageJ. Bounding regions for ipsilateral layers of the dLGN were automatically selected by filtering with a median filter (radius = 0.6 pixels) and a Gaussian filter (10 x 10 pixels) and autothresholding with the default settings of Fiji/ImageJ. The contralateral layer boundary of the dLGN (defined by CTB label) was manually traced. The positions of marked GFP-positive soma were then plotted onto the bounding regions using custom MATLAB software and cells within each bounded area/domain were manually counted.

Statistics

The net changes in branch tip lengths ($n\Delta L_{MD0}$, $n\Delta L_{MD3}$, and $n\Delta L_{MD7}$) were analyzed using mixed-effects models (R, nlme package) (Pinheiro J, Bates D, DebRoy S, Sarkar D and R Core Team (2016). *nlme: Linear and Nonlinear Mixed Effects Models*. R package version 3.1-128, http://CRAN.R-project.org/package=nlme) with a random intercept and slope for each animal for group comparisons over time. $n\Delta L_{MD0}$, $n\Delta L_{MD3}$, and $n\Delta L_{MD7}$ were used as the outcome measures for each group. Each repeat-measurement came from the same axon across days and included a grouping factor for axon to account for the correlation among each repeated measurement. A variance function was used to accommodate the different variation in measurements between groups. All other analyses were performed using Pearson's χ^2 test or the Wilcoxon rank-sum test in R (R Development Core Team (2008). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org). Significance was set at $p < 0.05$. All error bars are s.e.m..

Results

Labeling and imaging of eye-specific TC axons in V1

In adolescent mice, deprived-eye response depression induced by 3 days of MD is robust in the contralateral-eye pathway in mouse V1 and represents the earliest physiological change caused by deprivation, preceding open-eye response potentiation by several days (Frenkel and Bear, 2004). Therefore, we developed a preparation that allowed us to bias labeling of contralateral-eye TC axons so that we could monitor axonal structure and en passant bouton (EPB) turnover before and during MD.

We used a lentiviral vector to express membrane-bound GFP (mGFP) (Strobl et al., 2016)

in neurons located in the contralateral shell of the dorsal lateral geniculate nucleus (dLGN) (Fig. 1A) and in the region containing the binocular segment (Coleman et al., 2009). Contra- and ipsilateral laminae were delineated by differential labeling of retinal ganglion cell axonal inputs in dLGN with CTB-647 and CTB-555, respectively. Overall, sparsely labeled cells were largely restricted to the contralateral-eye layer in binocular (medial) dLGN (Fig. 1B-D) (contralateral-eye layer: 95 ± 0.02% (218/232 cells); ipsilateral-eye layer: 5 ± 0.02% (14/232 cells)). Labeled axon collaterals were readily visible in layer 1 and in some cases collaterals of the same arbor could be imaged down to layer 4 (data not shown), confirming that we were imaging axonal branches of geniculate projection neurons. In this study, we focused our efforts on tracking superficial-layer collaterals where we were able to consistently obtain detailed images of axonal branches/EPBs in layer 1. Prior to two-photon imaging sessions, intrinsic signal imaging was used to identify the binocular segment of V1, which is activated by both contra- and ipsilateral-eye stimulation (Fig. 2A). Overall, these results demonstrate that the experimental design (Fig. 2B) would ensure that imaging and analyses of labeled TC axons would be biased towards those subserving the contralateral (deprived) eye in binocular V1, where response depression is most robust and ocular dominance plasticity occurs.

Rapid pruning of TC axonal branches during MD

In mice, deprived-eye response depression is asymptotic within 3 days of MD and persists over longer periods of deprivation (Frenkel and Bear, 2004). We first confirmed that 3 days of MD beginning at P40 was sufficient to produce significant deprived-eye response depression as observed in mice with MD beginning at P25-P30 {Frenkel, Liu} (Fig. S1). Therefore, we imaged TC axonal branches in binocular mouse V1 every 3 to 4 days over 10 days, both prior to and during MD, which began around P40, and in non-MD control mice (Fig. 3A). Consistent with prior observations of steady-state TC axonal dynamics in somatosensory cortex (De Paola et al., 2006), overall TC axon structure was relatively stable over the course of imaging, but a small fraction of branch tips retracted and grew in V1 of non-MD control mice. Axonal branch tip length changes ranged from 5.1 to 21.7 µm (before baseline) and 4.3 to 31.9 µm (after baseline) in non-MD mice, and 4.7 to 22.1 µm (before baseline) and 4.2 to 156 µm (after baseline) in MD mice. Overall, there was no significant net change in branch tip length over the course of imaging in the non-MD control group (Fig. 3B) ($p = 0.77$, 95% CI = [-1.09, 0.81], linear mixed-effects (LME) model analysis; n = 43 branch tips from 4 mice). However, there was a significant decrease in branch tip length changes during MD ($p=0.007$, 95% CI = [-9.38, -1.52],

LME model analysis; n = 57 branch tips from 4 mice) (Fig. 3B). Furthermore, while there was no significant difference in branch length changes between groups during baseline imaging (non-MD = -0.73 ± 0.78 μm, MD = 0.35 ± 0.79 μm; $p > 0.05$, 95% CI = [-11.32, 4.13], LME model analysis), there was a significant difference during MD, which culminated in a net retraction of axonal branch tips of -8.54 ± 3.58 μm in MD mice versus -0.73 ± 0.70 μm in non-MD mice during 3 days of MD ($p < 0.05$, 95% CI = [-15.88, -2.50], LME model analysis), and -10.83 ± 3.71 μm in MD mice versus -1.01 ± 1.07 μm in non-MD mice during 7 days of MD ($p < 0.05$, 95% CI = [-22.51, -7.05], LME model analysis). In addition, we followed several branch tips in one subject out to 25 days post-MD (Fig. 3A). In an exceptional case, one small side branch extended ~20 μm between 3 and 7 days of MD and retracted back to its origin between 7 and 25 days of MD. In all other cases, branch tip retractions were only observed during 3-7 days of MD and were maintained after 25 days of MD. No extensions were observed during 7-25 MD. Overall, there were no changes associated with long-term MD (>7 days), thereby suggesting that branch pruning predominates during early MD.

Because a weakening and loss of deprived-eye TC synapses in V1 mark the earliest functional change during the OD shift, we reasoned that the direction of changes to TC axonal structural changes would match the direction of functional plasticity. Therefore, we divided the branch tips that showed changes in length greater than the measurement noise ($>3\sigma_{noise}$) into negative-going (retraction) and positive-going (extension) changes. There was no significant difference detected in the fraction of branch tips exhibiting retractions or extensions prior to (baseline: non-MD = 12% retractions/7% extensions, MD = 11% retractions/14% extensions; $p = 0.54$, χ^2 test) or during MD (3 days: non-MD = 19% retractions/14% extensions, MD = 28% retractions/14% extensions, $p = 0.53$, χ^2 test; 7 days: non-MD = 19% retractions/12% extensions, MD = 30% retractions/11% extensions, $p = 0.44$, χ^2 test). Prior to MD, there was no significant difference between groups in the magnitude of branch tip retractions (baseline: non-MD = 10.72 ± 2.82 μm, MD = 11.65 ± 2.25 μm; $p = 0.46$, rank-sum) or extensions (baseline: non-MD = 8.37 ± 2.00 μm, MD = 1.40 ± 1.01 μm; $p = 0.1$, rank-sum). However, there was a significant increase in the magnitude of branch tip retractions following 3 days of MD (non-MD = 7.67 ± 1.36 μm, MD = 32.97 ± 10.63 μm; $p = 0.014$, rank-sum), but not extensions (non-MD = 6.15 ± 0.83 μm, MD = 7.60 ± 1.00 μm ; $p = 0.37$, rank-sum) (Fig. 3C). Following 7 days of MD, there was no significant difference between groups in the magnitude of branch tip retractions (non-MD = 7.59 ± 1.19 μm, MD = 9.26 ± 1.50 μm; $p = 0.34$, rank-sum) or extensions (non-MD =

9.80 ± 2.11 µm, MD = 8.49 ± 2.63 µm; p = 0.46, rank-sum). Furthermore, the magnitude of branch tip retractions was significantly greater between 0-3 days of MD versus 3-7 days of MD (p = 0.02, rank-sum), but there was no significant change in the magnitude of branch tip extensions during this time (p = 0.70, rank-sum). There were no significant differences in the magnitude of retractions or extension of TC axon branch tips over the course of imaging for the non-MD group (0-3 days versus 3-7 days: retractions - p = 0.92, rank-sum; extensions - p = 0.20, rank-sum). Together, these data support the conclusion that large-scale changes to TC axonal structure are both temporally and directionally matched to deprived-eye response depression during 3 days of MD.

MD alters steady-state dynamics of EPB turnover

Previous two-photon imaging studies have shown that synaptic loss or gain can occur through branch tip remodeling and/or the turnover of EPBs (De Paola et al., 2006). In recent work, it was shown that synaptic turnover of EPBs intracortical axons is elevated in the somatosensory cortices of aged mice without detectable changes in branch tip remodeling (Grillo et al., 2013). Therefore, we next assessed whether the loss of TC synapses during MD was attributable exclusively to the remodeling of distal branch tips or whether EPBs along these same axonal regions were affected as well. We analyzed a total of 779 EPBs from both groups (non-MD = 469, n = 4 mice; MD = 310, n = 4 mice), of which an average of 71.74 ± 0.10% and 80.48 ± 0.10% were classified as EPBs (see Methods) and tracked from each mouse from non-MD and MD groups, respectively. In agreement with previous observations of TC axon EPBs in mouse somatosensory cortex (De Paola et al., 2006), the majority of TC axon EPBs in V1 were stable across both groups (0-3 days of MD: non-MD = 93 ± 1%, MD = 97 ± 2%; 3-7 days of MD: non-MD = 83 ± 3%, MD = 92 ± 1%), but a subset were lost or gained during imaging (Fig. 3D-E). There was no significant difference in the percentage of EPBs lost or gained between groups during the first 3 days of MD (EPB loss: non-MD = 7 ± 1%, MD = 4 ± 2 %, p = 0.34, rank-sum; EPB gain: non-MD = 4 ± 1%, MD = 3 ± 2%, p = 0.31, rank-sum) (Fig. 3D-E). By contrast, while the non-MD group showed a significant increase in EPB loss between 0-3 and 3-7 days (p = 0.03, rank-sum), there was no significant difference between time points for the MD group (p = 0.11, rank-sum). Furthermore, the percentage of EPB loss between 3-7 days of MD was significantly less than the non-MD control group (EPB loss: non-MD = 16 ± 2%, MD = 7 ± 2 %, p = 0.03, rank-sum). However, there were no significant differences in EPB gains between 3-7 days (non-MD 0-3 days versus 3-7 days, p = 0.89, rank-sum; MD 0-3 days versus 3-7 days, p =

0.66, rank-sum) or between groups (3-7 days non-MD versus MD, p = 0.89, rank-sum) (Fig. 3D-E). Together, these results show that steady-state losses and gains in EPBs during MD are not different during 3 days of MD, but that loss of EPBs is diminished during 3-7 days of MD.

Discussion

Although MD-induced changes in TC axon structure in V1 have served as a classic example of structural plasticity in the cortex, how this phenomenon is connected to rapid ocular dominance shifts has remained unclear. By showing that large-scale retractions of deprived-eye TC axonal branch tips are temporally matched to the time course of MD-induced response depression driven by these same inputs, we demonstrate that this form of structural plasticity is indeed fast enough to contribute to deprived-eye response depression in V1. This observation serves to bridge a gap between the rapid synapse-scale changes that accompany short-term MD and the large-scale anatomical changes to TC axons typically observed after long-term MD.

Antonini and Stryker (1993) demonstrated the earliest effects of MD on the terminal arbors of TC axons by showing significant pruning of individual deprived-eye axons after 7 days of MD in kitten V1. However, because pruning continued several days beyond the saturation of the physiological OD shift within 1-2 days of MD (Trachtenberg et al., 2000), the authors concluded these anatomical changes did not significantly contribute to changes in OD. Furthermore, similar work examining the effects of reverse suture in kittens showed that recovery of previously deprived eye responses occurred with no apparent recovery or growth of TC terminal arbors (Antonini et al., 1998). They went on to perform similar studies of TC terminal arbors in mouse V1, the results of which further reinforced the idea that structural plasticity of TC axons was too slow to contribute to the loss of visual function in the deprived-eye pathway. In mouse, the effects of long-term MD on TC axonal morphology were less pronounced than in cat with no evidence of branch retractions, even following 20-40 days of MD (Antonini et al., 1999). The only notable effect of MD as stated by the authors appeared to be an “arrest of the development of deprived-eye TC axons”, which in mouse still exhibit considerable growth between P40 and P60. By contrast, here we clearly demonstrate prompt retractions of TC axonal branches during 3 days of MD, which corresponds to the timing of maximal deprived eye response depression in mouse {Frenkel and Liu refs}.

Due to limitations of two-photon imaging, our study does not address the issue of whether changes to TC axon collaterals in deeper layers, especially layer 4, are more

pronounced or occur at all. It would be interesting to revisit the data obtained by Antonini et al. (1999) (Antonini et al., 1999) and determine whether layer-specific differences in branch lengths could be measured. One possible explanation for the apparently more subtle effects of MD on TC axons in mice versus cats is that mouse TC axons exhibit greater heterogeneity in their laminar branching pattern and terminal arbor density than those of cats or monkeys {REFs}. Thus, subtle differences in branch lengths in mouse may be beyond the detection limits of measurements made in static preparations. This fact highlights the advantage of our study where we were able to repeatedly image the same branch tips before and during MD, thereby making the detection of scant, cumulative changes in branch tip lengths possible.

Our findings strongly support the idea that the plasticity of TC axons is part of a continuum of circuit-level changes induced by MD rather than simply a long-term consequence. This is consistent with our previous work where we showed that TC synaptic loss and shrinkage of terminals occur in parallel with deprived-eye response depression in layer 4 of V1 (Coleman et al., 2010). However, whether these changes were coupled to deprived or non-deprived TC synapses could not be determined because we were unable to track eye-specific synapses. Here we addressed this issue by biasing lentiviral vector-mediated GFP label to deprived-eye TC axons (Fig. 1). We showed a rapid decrease in overall length of deprived-eye TC axonal branches during 3 days of MD. The fact that the only significant branch tip changes were retractions that coincided with 3 days of MD is consistent with a loss of TC input contributing to rapid synaptic weakening that occurs in layers 2/3 and 4 (Crozier et al., 2007; Liu et al., 2008; Rose et al., 2016). [[ROSE PAPER??]]

Importantly, while our observations were made in layer 1 rather than layer 4, it is likely that these changes reflect plasticity along the entire extent of the arbors. TC axonal EPBs in layer 1 of V1 reflect receptive field properties similar to those in layer 4 (Kondo and Ohki, 2016). In addition, a single TC axon can send collaterals to both layer 1 and layer 4 (Fig. S2) (Antonini et al., 1999). Further, restructuring of postsynaptic spines during MD occurs in other cortical layers, including layer 1. In fixed tissue, Mataga et al. (Mataga et al., 2004) reported a transient decrease in spine density in a region confined to a 25 µm-long segment of proximal apical dendrites of layer 2/3 pyramidal cells. Chronic two-photon imaging studies in V1 of Thy1-GFP mice revealed that 4 days of MD caused an increase in the motility of spines located on the apical dendrites of layer 5 pyramidal neurons, which is believed to reflect synaptic remodeling (Majewska and Sur, 2003; Oray et al., 2004). Interestingly, a recent two-photon imaging study showed no differences in EPB turnover on intracortical axons in layer 1 of V1 during 4-8 days of

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MD (Frantz et al., 2016), thereby suggesting that a the majority of dendritic spines in layer 1 that are responsive to MD may involve TC synapses. [[ROSE PAPER??]]

Our findings fit with a homosynaptic model of synaptic depression (Smith et al., 2009) where synaptic weakening/LTD-like synaptic plasticity of TC inputs is triggered by weak activation of NMDARs, which in turn triggers a series of biochemical events that culminate in the reduction of AMPARs and ultimately, synaptic transmission (Bear and Rittenhouse, 1999; Espinosa and Stryker, 2012). Interestingly, activity-dependent internalization of postsynaptic receptors precedes the subsequent withdrawal of the presynaptic terminal at the neuromuscular junction (Colman et al., 1997). Similarly, the physical withdrawal of TC axons/synapses from affected postsynaptic sites during MD may serve to consolidate plasticity linked to synaptic weakening, thereby suggesting the existence of a similar mechanism for central excitatory synapses.

It is important to note that mice do not seem to adhere to the classic 'critical period' for ocular dominance plasticity, but the kinetics of the ocular dominance shift do slow with age and it is unclear whether the same 'critical period' mechanisms are in play beyond P40/60 (Sato and Stryker, 2008). In adult mice, the same population of spines that show increased motility early in the critical period was shown to increase in density during 4-8 days of MD (Hofer et al., 2009), changes that presumably reflect structural correlates of non-deprived eye response potentiation that follows deprived-eye response depression (Frenkel and Bear, 2004; Frenkel et al., 2006; Hofer et al., 2006; Kaneko et al., 2008). However, because the identification of these synapses was not possible, the contribution of changes in postsynaptic spine density to the OD shift remains unclear.

TC axons provide input to a number of targets in layer 1, the majority of which are likely spines along the apical dendrites of pyramidal cells from layers 2/3, 4 and 5 {Bickford ref, rat layer 1}. While the effects of long-term MD may culminate in a more pronounced pruning of deprived-eye TC axons in additional layers of V1, our results suggest that rapid pruning of select branches that make up the complex TC axon terminal arbors in layer 1 contributes to early functional plasticity. Interestingly, we observed significant retraction of branch tips (and any synapses present along their length), but no significant loss of EPBs along spared regions of axon. While it is apparent from this study that overall deprived-eye branch tips are remarkably stable, the findings suggest that distal synapses are more vulnerable during MD and that they are lost in accord with their supporting branches. However, no differences in EPB losses or gains were observed between 0-3 days of MD, MD mice failed to show the degree of EPB loss

Comment [JC3]: 2012 LTD review; new Dudek paper

Comment [JC4]: Why is this para here?

Comment [JC5]: Mention EE effects?

exhibited by non-MD controls between 3-7 days of MD (Fig. 3E). The exact reason for this is not entirely clear, but it has been shown during development, synapses lost through axonal plasticity tend to be lost concurrently with branch retraction where branch tips retract to sites of EPB loss within a few hours (Ruthazer et al., 2006). By contrast, in adult neocortex, synaptic turnover tends to be dominated by changes in EPBs rather than large-scale retractions {REF}. Thus, the reduced EPB loss observed in the MD group may be a reversion mechanism to stabilize EPBs not lost to branch retractions in an attempt to preserve functional synapses. Together, our findings suggest a specific subpopulation of modifiable synapses is located at the distal tips of TC axons. It will be important to determine whether such changes are rapidly reversible or contribute to adult ocular dominance plasticity at all since brief MD-induced response depression can be rapidly reversed by normal visual experience (e.g., (Rose et al., 2016)) or pharmacological treatment (Fong et al., 2016), and if so, what mechanisms underlie reversible structural plasticity.

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Figure Legends

Figure 1: Lentiviral vector-mediated labeling of eye-specific TC axons with mGFP. (A) Schematic of the mouse visual system and injection regime. Top panel - Confocal image showing mGFP-labeled TC axons in binocular V1 (V1B). Bottom panel - Schematic showing the input zones for retinal ganglion cell axons from the contralateral (contra) and ipsilateral (ipsi) eyes into dLGN and locations of surrounding thalamic nuclei (lateral posterior nucleus (LPN), medial geniculate nucleus (MGN)). (B) Plots of mGFP-positive cells (green squares) throughout the injection zones of 3 mice. Yellow indicates the ipsilateral-eye projection zone shown in A. The dashed line delineates the LPN-MGN border. (C) Summary of the distribution of mGFP-positive cells within dLGN (n = 6 mice). (D) Summary of the distribution of mGFP-positive cells within dLGN and surrounding thalamic nuclei (same mice from C).

Figure 2: Chronic two-photon imaging of TC axons in V1B and experimental design. (A) Cartoon showing a dorsal view of mouse brain indicating the location of the cranial window relative to V1. To the right and below are heat maps of visually evoked intrinsic signals and the threshold of the ipsilateral-eye map superimposed over the vasculature map, respectively. The solid orange border indicates the area subjected to repeated two-photon imaging. (B) An example two-photon image showing a best maximum-intensity projection of four Z-stacks stitched together. The inset shows a portion of an axon arbor branch traced to its tip and segmented using the fill-out feature of the simple neurite tracer plug-in in Fiji. The segmented branch is shown as a maximum-intensity projection of all optical sections through the entire Z-stack, which is how all axon branch images are presented in the other figures.

Figure 3: Structural plasticity of TC axonal arbors and EPBs in layer 1 during MD. (A) Example maximum-intensity Z-projections of segmented axonal arbors in layer 1 from an MD animal. The last panel is an image acquired during 25 days of MD, showing that most of the structural changes occurred within the first 3-7 days of MD. Green arrowheads = branch retraction, magenta arrowheads = branch extension. (B) Summary of branch tip dynamics during MD (non-MD control, n = 43 branch tips from 4 mice; n = 57 branch tips from 4 mice, * $p < 0.05$). (C) Summary of branch tip retractions and extensions within each imaging interval that were greater than the measurement noise (* $p < 0.05$). Scale bar: 50 µm. (D) Example two-photon images of TC axonal EPBs that were stable, lost, or gained during imaging. (E) Summary of EPB losses

and gains during MD (* $p < 0.05$).

Figure S1: Robust MD-induced deprived-eye response depression in P40 wild-type C57Bl/6 mice. (A) Experimental design. As previously described (Frenkel and Bear, 2004), mice were chronically implanted with an electrode in V1B at P36. Following habituation to the recording apparatus (P37–39), baseline visually evoked potentials (VEPs) were recorded in awake mice at P40 and mice underwent MD for 3 days. At P43, the eye was opened and VEPs recorded again. (B) MD produced a depression of contralateral-eye responses (blue bars), but no change in ipsilateral-eye responses (yellow bars; n = 3 mice, * $p < 0.05$, paired t-test day 0 vs. day 3).

Figure S2: Three-dimensional reconstruction of a partial TC axon from a 2-photon image Z-stack obtained *in vivo*. Note that the single axon sends collaterals to layers 1 and 4, which are shown as XY maximum-intensity Z-projections (i.e. bird's eye view) shown in cyan and red boxes.