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Increased Axonal Bouton Stability during Learning in the Mouse Model of MECP2 Duplication Syndrome

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Title: Increased axonal bouton stability during learning in the mouse model of MECP2 duplication syndrome

Abbreviated title: Bouton hyperstability in MECP2 duplication syndrome

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ABSTRACT

MECP2-duplication syndrome is an X-linked form of syndromic autism caused by genomic duplication of the region encoding Methyl-CpG-binding protein 2. Mice overexpressing *MECP2* demonstrate social impairment, behavioral inflexibility, and altered patterns of learning and memory. Previous work showed abnormally increased stability of dendritic spines formed during motor training in the apical tuft of primary motor cortex (area M1) corticospinal neurons in the MECP2-duplication mouse model. In the current study, we measure the structural plasticity of axonal boutons in Layer 5 (L5) pyramidal neuron projections to layer 1 of area M1 during motor training. In wild-type littermate control mice we find that during rotarod training, bouton formation rate changes minimally, if at all, while bouton elimination rate more than doubles. Notably, the observed upregulation in bouton elimination with training is absent in *MECP2*-duplication mice. This result provides further evidence of an imbalance between structural stability and plasticity in this form of syndromic autism. Furthermore, the observation that axonal bouton elimination more than doubles with motor training in wild-type animals contrasts with the increase of dendritic spine consolidation observed in corticospinal neurons at the same layer. This dissociation suggests that different area M1 microcircuits may manifest different patterns of structural synaptic plasticity during motor training.

SIGNIFICANCE STATEMENT

Abnormal balance between synaptic stability and plasticity is a feature of several autism spectrum disorders, often corroborated by in vivo studies of dendritic spine turnover. Here we provide the first evidence that abnormally increased stability of axonal boutons, the presynaptic component of excitatory synapses, occurs during motor training in the *MECP2* duplication syndrome mouse model of autism. In contrast, in normal controls, axonal bouton elimination in L5 pyramidal neuron projections to layer 1 of area M1 more than doubles with motor training. The fact that axonal projection boutons get eliminated, while corticospinal dendritic spines get consolidated with motor training in layer 1 of

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area M1, suggests that structural plasticity manifestations differ across different M1 microcircuits.

INTRODUCTION

The rewiring of synaptic connections in neural microcircuits provides a compelling mechanism for learning and memory throughout development and adult life (Chklovskii et al., 2004). Two-photon imaging of fluorescently-labeled neurons has recently enabled the direct measurement of synaptic rewiring in vivo, revealing that new synapses form in motor cortex (M1) during motor training, and that the stability of these synapses correlates with how well the animal learns to perform the motor task (Xu et al., 2009; Yang et al., 2009). The layer 1 (L1) apical tuft dendritic spines that turn over during training receive inputs from a range of sources, including L2/3, L5, and L6 cortical pyramidal neurons, thalamocortical neurons, and others. It is currently not known how synaptic inputs from axonal projections to area M1 behave during training.

Experimental LTP and LTD paradigms in vitro can induce axonal bouton formation and elimination (Antonova et al., 2001; Becker et al., 2008; Bourne et al., 2013). In vivo, axonal boutons are spontaneously formed and eliminated in adult sensory cortex (De Paola et al., 2006; Majewska et al., 2006; Stettler et al., 2006; Grillo et al., 2013), while behavioral training has been shown to alter bouton turnover in parallel fiber inputs to the cerebellum (Carrillo et al., 2013) and in orbitofrontal inputs to the medial prefrontal cortex (Johnson et al., 2016). In this work we examine the turnover of boutons, the pre-synaptic component of synapses, in L5 pyramidal neuron axons that project to layer 1 of area M1.

Furthermore, we begin to assess whether training-associated plasticity in inputs to area M1 is altered in the *MECP2*-duplication model of autism. *MECP2* duplication syndrome is caused by a genomic duplication that spans the methyl-CpG-binding protein 2 (*MECP2*) gene and leads to a progressive X-linked disorder of intellectual disability, autism, spasticity, and epilepsy (Ramocki et al., 2010). Overexpression of the *MECP2* gene in mice produces a similar progressive neurological phenotype including autistic features (abnormal social behavior, anxiety, and stereotypies), spasticity, and epilepsy (Collins et al., 2004), and abnormal dendritic structure and plasticity (Jiang et al., 2013). Previous work found an

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increase in the formation and stabilization of dendritic spine clusters in apical dendritic tufts of corticospinal neurons in M1 (Ash et al., 2017) in these mice, pointing to a possible abnormal imbalance between synaptic stability and plasticity.

MeCP2 and other autism-associated proteins contribute to the development of mature axons and presynaptic structures (Antar et al., 2006; Belichenko et al., 2009; Degano et al., 2009; Chen et al., 2014; Garcia-Junco-Clemente and Golshani, 2014). Presynaptic electrophysiological function has been shown to be altered in *MECP2*-duplication mice (increased paired pulse facilitation, Collins et al., 2004) and other autism mouse models (Deng et al., 2013), and mice with mutations in the proteins mediating presynaptic plasticity often demonstrate autistic features (Blundell et al., 2010). Long term depression (LTD), a form of synaptic weakening that has a major pre-synaptic component (Collingridge et al., 2010), has been shown to be defective in several models of autism (D'Antoni et al., 2014). These findings implicate pre-synaptic dysfunction in autism, but axonal bouton structural plasticity has not been explored directly in a model of autism to our knowledge.

We measured axonal bouton structural plasticity in layer 1 of mouse M1 during rotarod training in the Tg1 mouse model of the *MECP2* duplication syndrome and compared with wild-type (WT) littermates. We found that the rate of bouton formation does not change significantly with rotarod training in either genotype, remaining approximately the same as the spontaneous bouton formation rate at rest. In contrast, bouton elimination rate is dramatically accelerated during rotarod training in WT mice, whereas this effect is completely abolished in *MECP2*-duplication mice. This supports the argument that increased synaptic stability manifests in the MECP2-duplication syndrome during training (Ash et al., 2017).

MATERIALS & METHODS

Animals. FVB-background *MECP2*-duplication (Tg1) mice (Collins et al., 2004), were crossed to C57 thy1-GFP-M (Feng et al., 2000) homozygotes obtained from Jackson Laboratories, to generate male F1C57;FVB *MECP2*-duplication;thy1-GFP-M mice and thy1-GFP-M littermate controls. All animal procedures were performed in accordance with the Baylor College of Medicine animal care committee's regulations.

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In vivo two-photon imaging. All surgeries and imaging were performed blind to genotype. At least two weeks prior to the first imaging session (~12-14 week-old-mice), a 3 mm-wide opening was drilled over motor cortex, centered at 1.6 mm lateral to bregma (Tennant et al., 2011), and a glass coverslip was placed over the exposed brain surface to allow chronic imaging of neuronal morphology (Mostany and Portera-Cailliau, 2008; Holtmaat et al., 2009; Mostany et al., 2013). Neural structures were imaged using a Zeiss *in vivo* 2-photon microscope with Zeiss 20x 1.0 NA water-immersion objective lens. High-quality craniotomies had a characteristic bright-field appearance with well-defined vasculature and pale grey matter (Fig. 1A). Under two-photon scanning fluorescent structures were reliably clear and visible with low laser power (<20 mW). A 0.1 micron diameter fluorescent bead acquired with our 2-photon imaging set up is ~0.4 microns full width at half-maximum (Fig. 1B), confirming that our resolving power is sufficient to distinguish the 1-3 micron diameter boutons we followed in the study. Only high quality preparations (low background noise across all time points, <5 pixel i.e. <0.5μm slow motion artifact, <2 pixel i.e. <0.2 μm fast motion artifact, and axons well isolated from other fluorescent structures) were used in the blinded analysis. Pyramidal neuron axons were imaged at high resolution (310x310 to 420x420 μm FOV, 0.1 μm/pixel, 1 μm Z-step size) to adequately capture individual boutons. Laser power was maintained under 20 mW (average ~10 mW) during image stack acquisition.

Motor training. The Ugo Basile mouse rotarod was used for motor training. At least two hours after imaging sessions, in the late afternoon, mice were placed on the rotarod, and the rotarod gradually accelerated from 5 to 80 rpm over 3 minutes. Single-trial rotarod performance was quantified as the time right before falling (16 cm fall height) or holding on to the dowel rod for two complete rotations without regaining footing. A 7-10 minute rest period occurred between each trial. Four trials were performed per day.

Analysis of bouton plasticity. Analysis was performed blind to genotype. Axons were chosen from the imaging field based on characteristic appearance, including the absence of dendritic spines, minimal branching, and the presence of synaptic boutons, as well as decreased width compared to dendrites. In the thy1-GFP M mouse line (Feng et al., 2000) we employed, the vast majority of GFP-labeled axons in the cerebral cortex

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159 arise from L5 pyramidal neurons, though occasional L2/3, L6 pyramidal neurons and
160 thalamocortical neurons may also be labeled (De Paola et al., 2006). Pyramidal neuron
161 axons were targeted based on their thin shafts, high density of small (<3 μm diameter)
162 en-passant boutons, low tortuosity, and rare branching (type A3 axons), allowing them
163 to be clearly distinguished from i) L6 pyramidal neuron axons, which have high
164 branching and a high density of terminaux boutons, and from ii) thalamocortical
165 neurons, which have thicker axons and high branching (De Paola et al., 2006). Given
166 the very sparse labeling of L2/3 neurons in the thy1-GFP M mouse line, we are
167 confident that the great majority of axonal segments we imaged represent L5 pyramidal
168 neuron projections to area L1 from other regions, i.e. chiefly from the premotor, the
169 somatosensory and the contralateral motor cortex (Hooks et al., 2013).

170 Segments of axon that were clearly visualized in all three time points were selected for
171 analysis (length range 30 – 360 μm, mean 138 μm). The presence of en-passant
172 boutons or terminaux boutons was noted by a blinded investigator, who further
173 classified synaptic boutons as alpha (> ~2 μm or 20 pixel diameter) or beta (<~2 μm or
174 20 pixel diameter). The threshold used for bouton classification was based on the
175 bimodal distribution of boutons, separable at ~2 μm diameter, present in the analyzed
176 data set (Fig. 1C, Grillo et al., 2013). The presence of a bouton was determined by a
177 clear increase in axon diameter, increased fluorescence compared to the background
178 axon, and the characteristic varicose contour determined by the judgment of an
179 experienced investigator. In general, varicosities counted as boutons were >3 pixels
180 (~0.3 microns) wider than the axonal shaft diameter (corresponding to approximately to 2
181 SDs of the noise blur of the axonal shaft, see Fig.1 B), and more than twice as bright as
182 the axonal backbone, as in (Grillo et al., 2013).

183 Boutons located greater than 50 μm away from the nearest other bouton were excluded
184 from the analysis, so that stretches of bouton-free axon would not bias bouton density
185 calculations. Four to twenty axons were analyzed from 1-3 imaging fields per mouse for
186 13 mice (6 WT, 7 *MECP2*-duplication mice). Unless the investigator could clearly trace
187 the continuity of axon segments, segments were analyzed as individual units. Though
188 unlikely, the possibility cannot be completely excluded that, on occasion, more than one

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segment from a single axon were counted. Bouton formation and elimination (Fig. 2B, 3A,B) was calculated as (boutons formed or boutons eliminated) / (total number of boutons observed across imaging sessions), analogous to the measure used in (Grillo et al., 2013). Bouton survival was calculated as the percent of boutons identified in the first imaging time point that are present in subsequent imaging time points. Bouton stabilization was calculated as the percent of newly formed boutons in the second imaging time point, which persisted in the third imaging time point.

***Statistics*.** Except where indicated, the Mann-Whitney U test was used for two-group statistical comparisons, and the linear mixed-effects models ANOVA was used for multi-group comparisons. The linear mixed-effects model ANOVA was instantiated with genotype and imaging time point as fixed effects and mouse and axon implemented as random effects. This approximates a repeated-measures ANOVA for the 2-way experimental design, accounting for any across-animal variability in determining statistical significance.

RESULTS

The Tg1 mouse model for *MECP2* duplication syndrome (FVB background) was crossed to the thy1-GFP-M mouse line (C57 background) to generate F1 hybrid males for experiments. A cranial window was placed over motor cortex (1.6 mm lateral to bregma) at 12-14 weeks of age, and at least 2 weeks following the surgery the mouse was placed under the 2-photon microscope to image GFP-labeled axons in layer 1 of area M1 (Fig. 1A; see methods).

L5 pyramidal neuron axons are typically visualized as a thin string of fluorescence interspersed with fluorescent expansions or varicosities (en passant boutons) and rare spine-like terminaux boutons. They are readily differentiated morphologically from L6 neuron axons and thalamocortical axons (De Paola et al., 2006), which, in any case, are rarely fluorescent in these animals. The thy1-GFP M line primarily labels L5 pyramidal neurons in neocortex, and therefore the majority of axonal arbors we imaged are expected to arise from L5 of the somatosensory cortex, the premotor cortex, or the contralateral motor cortex, all of which project to L1 of area M1 (Colechio and Alloway,

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2009; Mao et al., 2011; Hooks et al., 2013). Area M1 L5 neurons rarely send projections locally to layer 1 (Cho et al., 2004).

First, we report on axonal bouton structure and plasticity analyzed in littermate controls with normal *MECP2* expression. Axonal boutons were identified as periodic thickenings or extensions along the axon (Fig. 1B, see Methods). We observed a bimodal distribution of bouton sizes, the two modes separated at approximately 2μm diameter (Fig. 1C). These large (alpha) and small (beta) boutons were analyzed separately. The density of alpha boutons was 2.7±0.3 boutons/100μm (mean±SEM, n=58 axonal segments), and the density of beta boutons was 4.0±0.4 boutons/100μm (Fig. 1D,E), similar to a previous study (see Methods, Grillo et al., 2013). As expected given their large size (Grillo et al., 2013), alpha boutons were much more stable than beta boutons (Fig. 1F). Across 4 days of rest the 4-day turnover rate (TOR = (gain rate+loss rate) /2) of alpha boutons was 0.5±0.25% (0.02±0.01 boutons/100μm), while the TOR of beta boutons was 23±4% (0.59±0.08 boutons/100μm). These results are comparable to a previous study in somatosensory cortex, which found 0.1±0.06% 4-day turnover for large boutons and 30±3% 4-day turnover for small boutons (see Fig. 4E,F in Grillo et al., 2013). Since alpha boutons were stable over time, hardly changing over the time course of the experiment, we restricted further analysis of structural plasticity to beta boutons.

The experimental design is diagrammed in Fig. 2A. L5 pyramidal neuron axonal projections to layer 1 (L1) of area M1 were initially imaged to identify baseline boutons. Then mice underwent four days of training on the accelerating rotarod task. Axons were re-imaged to quantify training-associated bouton turnover. Mice rested in the home cage for four days, and axons were imaged again to observe bouton turnover during rest. WT mice performed progressively better on the rotarod across 4 days of training as reported before (Buitrago et al., 2004; Collins et al., 2004). Interestingly, rotarod training led to a dramatic increase in bouton elimination compared to rest: 17±3% of total beta boutons were lost after 4 days of training compared to 6±2% of total boutons lost after 4 days of rest (Fig. 2B, p=0.001, Mann-Whitney U test, n=58 axon segments from 6 mice). Bouton formation rate, in contrast, did not change significantly during motor training (Fig. 2B; training: 10±2% of total boutons across time points, rest: 9±2% of total

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boutons, p=0.5). The measured formation rates and elimination rates were comparable to the spontaneous 4-day bouton formation and elimination rates previously observed in L5 pyramidal neuron axons in somatosensory cortex (formation: 8±1%, elimination: 8.0±0.2%, Fig. S4C,D in Grillo et al., 2013). Overall, in control animals, motor training induces a doubling of bouton elimination in M1 without a concomitant change in the rate of bouton formation.

Plotting the survival fraction of pre-existing ("baseline") boutons revealed that L5 pyramidal axons projecting to L1 of area M1 maintained 77±4% of their baseline boutons (boutons present pre-training, on day 0) through 4 days of training (Fig. 2C). This value is significantly lower than prior estimates of spontaneous 4-day survival fraction of L5 pyramidal neuron axonal boutons (~90% of baseline boutons, dotted line in Fig. 2C, see Fig. 7B of De Paola et al., 2006, Fig. 3C of Grillo et al., 2013, Fig. 5 of Majewska et al., 2006).

Note that elimination rates (Fig. 2B) and survival curves (Fig. 2C) do not sum exactly to 100% because elimination rate was calculated as a fraction of the total number of beta boutons observed across all time points to avoid outlier turnover rates in axons which had very few baseline boutons, following Grillo et al., 2013 (see Methods).

We also compared the survival rate of newly formed training-related boutons with that of pre-existing boutons. In the four days of rest following training, 85±4% of baseline preexisting boutons (boutons present on day zero that were also present on post-training day 4) were maintained, while newly formed boutons were maintained at a much lower rate of 32±9% (Fig. 2D, $p=10^{-6}$, Mann-Whitney U test), consistent with the reported stabilization rate of spontaneously formed boutons in somatosensory cortex (newly formed: 35±5% of all boutons over 4 days, Grillo et al., 2013).

We then assessed training-associated axonal bouton turnover in *MECP2*-duplication mice. *MECP2*-duplication mice performed significantly better on the rotarod than control littermates as previously described (Collins et al., 2004; Ash et al., 2017). The average length of analyzed axonal segments was not significantly different between mutants and WT littermates (WT: 142±73 µm, *MECP2*-duplication: 133±73 µm, mean±SD). The density of alpha boutons (Fig. 1D) and beta boutons (Fig. 1E) was also similar between the genotypes (*alpha boutons,* control: 2.7±0.3 boutons/100µm,

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MECP2-duplication: 2.4±0.3 boutons/100µm, p=0.4; *beta boutons*, control: 4±0.4 boutons/100µm, *MECP2*-duplication: 5.8±0.7 boutons/100µm. p=0.2, Mann-Whitney U test). Similar to WT, alpha boutons were highly stable compared to beta boutons in *MECP2*-duplication mice (Fig. 1F).

Interestingly, the increased bouton elimination rate during training observed in WT mice did not occur in *MECP2*-duplication mice (Fig. 3B). Significantly fewer boutons were eliminated during training in *MECP2*-duplication mice (Fig. 3B, training: 5±1% of total beta boutons; rest: 4±1% of total boutons; n=54 axon segments from 7 mice) compared to littermate controls (training: 17±3%, rest: 6±2% of total boutons; n=58 axon segments from 6 mice; effect of genotype: t=-2.9, p=0.003; effect of training vs. rest: t=-3.5, p=0.0004; genotype x training interaction: t=2.6, p=0.009; linear mixed-effects models ANOVA, see Methods). Plotting the survival fraction of baseline (pretraining) boutons revealed that baseline boutons were significantly more stable in *MECP2*-duplication mice vs. littermate controls, especially during training (Fig. 3C, Effect of genotype: t=-2.8, p=0.004; effect of training vs. rest: t=-3.1, p=0.002; genotype x training interaction: t=2.5, p=0.01). *MECP2*-duplication axons maintained 95±1% of their boutons after 4 days of training, while control littermate axons maintained only 77±4%. *MECP2*-duplication axons lost a further 6±1% of baseline boutons to reach 89±2% bouton survival on day eight, while littermate controls lost a further 8±2% to end at 69±4%.

The rate of beta bouton formation was not significantly different between *MECP2*-duplication mice and WT controls, neither during the training (Fig. 3A, control: 12±2% of total boutons; *MECP2*-duplication: 10±2% of total boutons) nor during the rest phase (control: 9±2 % of total boutons, *MECP2*-duplication: 6±1 % of total boutons, effect of genotype: t=0.5, p=0.6; effect of training vs. rest: t=0.4, p=0.6; genotype x training interaction: t=-0.8, p=0.4). The stabilization rate of newly-formed boutons was also not significantly altered in *MECP2*-duplication mice (40±8%) compared to controls (32±9%, Fig. 3D, p=0.3). Again, note that elimination rates (Fig. 3B) and survival curve percentages (Fig. 3C) do not sum to 100%, as explained above, but note that the

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measured differences remain significant if the elimination rate is calculated as a fraction of baseline boutons instead of as a fraction of total boutons across time points (Fig. 3C).

Bouton formation, elimination, and stabilization rates did not correlate well with rotarod performance in individual animals for either genotype or pooled across genotypes ($p>0.05$, t-test on linear regression, all comparisons, data not shown), suggesting that other factors are potentially more important for the behavioral manifestations of motor learning.

DISCUSSION

The stability and plasticity of synaptic connections is a tightly regulated process that unfolds throughout life. A pathological imbalance between stability and plasticity could lead to the altered patterns of learning and forgetting observed in autism mouse models (Collins et al., 2004; Rothwell et al., 2014) and in autistic patients (Treffert, 2014). In prior work (Ash et al., 2017) an abnormal increase in training-associated dendritic spine stability was found in the apical tuft of area M1 corticospinal neurons in the Tg1 mouse model of *MECP2* duplication syndrome. Here we investigated how axonal boutons in the L5 pyramidal neuron projection to L1 of primary motor cortex turn over during motor training in these animals. First, we find in WT mice that: **1)** bouton formation rate is unaffected by motor training (Fig.2B), and **2)** bouton elimination rate more than doubles from ~6% to ~17% during training (Fig. 2B,C). In contrast, we find that the increase in training-associated bouton elimination observed in littermate controls does not occur in *MECP2*-duplication mice (Fig. 3B), which exhibit increased bouton stability, particularly during training (Fig. 3C). Bouton formation rate during motor training was similar between *MECP2*-duplication animals and littermate controls (Fig. 3A), and was not significantly different from the rate of bouton formation observed at rest in either genotype. A similar fraction of training-associated boutons was stabilized in both genotypes (Fig. 3D).

Bouton formation and elimination with motor training in controls

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Our spontaneous 4-day bouton turnover results are in agreement with a previous study of axonal bouton formation and elimination in L5 pyramidal neuron axons projecting to layer 1 of somatosensory cortex (Grillo et al., 2013), suggesting that baseline axonal bouton turnover in L1 is similar in sensory and motor areas. Here, we found that, in normal animals, the rate of axonal bouton elimination increases markedly during motor training in L5 pyramidal neuron projections to L1 of area M1, without a concomitant increase in the rate of bouton formation (Fig. 2B).

Grillo et al. 2013 performed post-hoc electron microscopy reconstructions of nine axonal varicosities detected by 2-photon and found that all nine boutons formed synapses, suggesting that the great majority of 2-photon-identified boutons form a synapse. Our results therefore suggest that training leads to a weakening of L5 pyramidal inputs to layer 1 of area M1, at least as evidenced by structural analysis. Layer 5 axonal projections to L1 have several potential synaptic partners, including apical dendritic arbors of L5B corticospinal pyramidal neurons, L5A corticostriatal/corticocallosal neurons, L2/3 pyramidal neurons, and L1 interneuron dendrites (Fig. 4). Since L1 interneurons are sparse, most of the postsynaptic partners of the axonal boutons we studied are likely formed with one or more of the aforementioned classes of pyramidal neurons.

The increased elimination of pre-synaptic axonal boutons during training would then lead us to expect a corresponding loss in their post-synaptic partners, i.e. of dendritic spines located in the apical dendritic tufts of the target neurons. However, an increase in the formation rate of dendritic spines has been previously shown during motor training in the apical tuft terminal dendrites of L5 neurons in layer 1 of area M1 (Xu et al., 2009; Yang et al., 2009). This dissociation between L5 neuron dendritic spine formation and axonal bouton elimination during motor training suggests that the pre-synaptic partners of the L5 apical tuft dendritic spines studied previously during motor learning (Xu et al., 2009; Yang et al., 2009) arise from thalamocortical, L2/3, or L6 projections, which we did not study here. Indeed, projections to L1 of M1 from different brain areas and layers are known to preferentially target different cell types (Hooks et al., 2013).

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Another nonexclusive possibility is that rather than connecting with a new axonal bouton, newly formed spines form a second synapse onto large pre-existing boutons already harboring a synapse. Evidence for this comes from correlative electron microscopy studies in the somatosensory cortex and hippocampus: ~70% of newly formed spines synapse with a multi-synapse bouton, compared to 20-30% of preexisting spines (Knott et al., 2006; Nagerl et al., 2007); see also (Woolley et al., 1996; Toni et al., 1999; Geinisman et al., 2001; Yankova et al., 2001; Federmeier et al., 2002; Nicholson and Geinisman, 2009; Lee et al., 2013). Dendritic spines formed during training may largely synapse on already existing, large, pre-synaptic boutons (alpha boutons in our study) where they compete with the previously present connections. Over time, some of these connections withdraw, re-establishing a new equilibrium that favors the new skill learning. Presumably, in the days-to-weeks following training, bouton formation modestly increases and/or bouton elimination decreases to bring bouton densities back to baseline levels. Overall, these results raise the interesting possibility that different pathways projecting to L1 of mouse area M1 may have different signatures of structural plasticity during motor learning.

Increased bouton stability in *MECP2*-duplication mice

We found that the training-associated increase in bouton elimination rate occurring in WT mice is abolished in *MECP2*-duplication mice. The simplest interpretation of these results is that the L5 pyramidal neuron projection to L1 of area M1 undergoes less synaptic reorganization during training in mutants. In this case, the elevated synaptic turnover seen in mutant M1 (Ash et al., 2017) must be occurring in other L1 sub-circuits (e.g. L2/3 or L6 pyramidal neuron projections). Increased bouton stability could also be due to more robust capture and stabilization of pre-existing boutons by newly formed training-associated spines, boutons that would have otherwise been eliminated due to loss of their prior post-synaptic targets during the training period (Knott et al., 2006; Nagerl et al., 2007). In this case, it would be possible to have accelerated reorganization in synaptic connectivity in the L5 pyramidal neuron to L1 circuit projection without any measurable change in the turnover of boutons. Imaging of bouton turnover

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in other projections to L1 of area M1 and quantification of multi-synapse bouton density with and without training in mutants could address these two possibilities.

It is interesting to speculate that the training-associated bouton elimination that occurs in littermate controls is a natural end result of strong long-term depression (Becker et al., 2008; Wiegert and Oertner, 2013). In this case, the lack of bouton elimination in mutants may connote a disruption in processes regulating LTD. Taken along with the fact that abnormal LTD is observed in many other autism models (D'Antoni et al., 2014), it will be interesting to experimentally test if LTD is indeed altered in M1 of *MECP2*-duplication mice, and to see if decreased LTD underlies the mutant's increased learning-associated bouton stability.

Relationship between bouton turnover and learning

The behavioral implications of increased L1 axonal bouton stability in mutants remain a matter of speculation. In our motor training experiments bouton elimination did not strongly correlate with behavioral performance either in mutants or controls, suggesting that other factors are potentially more important for the behavioral manifestations of motor learning. Prior work has shown that apical tuft L5 pyramidal neuron dendritic spine formation correlates with motor learning in normal animals (Yang et al., 2009), and *MECP2*-duplication animals are known to exhibit increased spine formation and stabilization during learning (and at baseline) compared to wild-type littermates (Jiang et al., 2013; Ash et al., 2017).

We hypothesize that increased bouton survival during this period may in part reflect a higher rate of synapse stabilization, possibly due to an increased ability of *MECP2*-duplication boutons to form synapses with newly generated spines. Although we have not proven this here, this may contribute to the faster and more durable learning that *MECP2*-duplication animals exhibit in simple tasks like the rotarod and conditioned fear memory (Collins et al., 2004). Over time however, the same process may restrict the overall flexibility of the motor circuit, leading to the motor deterioration phenotype observed at later ages.

Potential Limitations

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It is important to note a number of limitations with the study. First of all, our quantification of presynaptic terminals depends entirely on morphological measures. We used conservative criteria similar to that which in prior experimenters' hands have been shown to reliably detect synapse-forming puncta (De Paola et al., 2006), and a 2-photon study that systematically correlated bouton diameter with presence of an EM-verified synapse found that all nine boutons they studied formed synapses, even the smallest, which had a diameter ~0.4 µm, considerably smaller than the boutons we study here (range 1-3 µm, Fig. 1C) (Grillo et al., 2013). This suggests that the great majority of boutons we identify by 2-photon form a synapse. Prior studies of bouton ultrastructure have estimated that ~10% of varicosities do not form a synapse (Shepherd and Harris, 1998; White et al., 2004; Bourne et al., 2013), but these studies included also smaller varicosities and none related axonal varicosity size to the probability of a synapse to our knowledge.

Second, the rest phase occurred following training, so it is possible that some of the corresponding bouton turnover may reflect enduring consolidation processes that persist beyond training rather than a true rest phase. Having said that, the measured spontaneous axonal bouton formation and elimination is in very close agreement to previous studies (Grillo et al., 2013), suggesting that the measurements reflect baseline turnover.

Third, we cannot precisely determine the origin of the axonal afferents imaged in our study (Fig. 4). Some of the heterogeneity in plasticity observed across imaged axons could be due to projection-specific differences. For example, it would be interesting to speculate that coarse sensorimotor training induced by the rotarod may drive greater bouton remodeling in somatosensory cortical inputs to area M1, while fine motor training requiring higher-order motor planning, such as the seed-grabbing task used by (Xu et al., 2009), may induce greater remodeling in premotor cortical inputs.

Fourth, the postsynaptic partners of the imaged axons are unknown. The precise connectivity of inputs to M1, with S1 pyramidal neuron axons preferentially synapsing on L2/3 and L5A neurons and premotor cortex pyramidal neuron axons preferentially synapsing on L5B neurons (Mao et al., 2011; Hooks et al., 2013), enables a rich potential repertoire of synaptic reorganization during training. New methods targeting

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fluorescent proteins to specific input areas, as well as combinatorial techniques labeling pre-and postsynaptic partners (Kim et al., 2011; Druckmann et al., 2014), will be needed to tackle this question in the future.

Conclusions and implications

In conclusion, we report here that L5 pyramidal neuron axonal projections to layer 1 of WT mouse motor cortex exhibit a selective escalation in bouton elimination during motor training, a plasticity process that is disrupted in the *MECP2*-duplication syndrome mouse model of autism. These data constrain models of motor cortex plasticity underlying learning and underscore the possibility that different synaptic pathways within the cortical circuit may manifest different patterns of structural synaptic plasticity during learning. Future work studying plasticity along different synaptic pathways that link various areas along the motor circuit will shed further light on these issues. Finally, our results provide further evidence for an altered balance between stability and plasticity of synaptic connections in favor of stability in the *MECP2* duplication syndrome mouse model.

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FIGURE LEGENDS

Figure 1 - Bouton classification and density of L5 pyramidal neuron axonal projections to layer 1 of mouse primary motor cortex. (**A**): In vivo 2-photon imaging. (1) A cranial window is drilled centered 1.6 mm lateral to the bregma to expose area M1. Correct localization to the forelimb was confirmed post-hoc by electrical microstimulation; see (Ash et al., 2017). (2) GFP-labeled pyramidal neuron processes in layer 1 of area M1 are imaged. Yellow box shown at high zoom in panel B. **(B)**: *Top*: 2-photon image of a 0.1 micron diameter fluorescent bead, revealing the resolving power of the microscope to be 0.4 µm full width at half-maximum. *Bottom*: Example small (1.2 µm diameter) bouton at the same magnification for comparison, showing that the microscope's resolution allows ready discrimination of the boutons in this study. (**C**): Bouton classification. *Left:* Varicosities along axons are classified as alpha (>~2 µm diameter, blue arrows) or beta (1-2 µm diameter, yellow arrows) boutons based on size (see Methods). Extraneous fluorescence structures masked for illustration purposes only. *Right:* Histogram of bouton diameters measured in a subset of axons (n=54 alpha, 74 beta boutons), demonstrating a bimodal distribution. (**D,E**): Histogram of densities of alpha (**D**) and beta (**E**) boutons per axonal segment in *MECP2*-duplication mice (orange, n=54 segments from 7 mice) and WT littermates (black, n=58 segments from 6 mice). (**F**): four-day spontaneous bouton turnover rate, (boutons formed + boutons eliminated) / 2*axon length, for alpha boutons and beta boutons. Alpha boutons were highly stable in this time frame.

Figure 2 - Bouton elimination increases during motor training in L1 of WT motor cortex (**A**): Experimental paradigm and imaging time points. Sample images of axonal segments imaged before (left) and after (middle) 4 days of rotarod training to identify axonal bouton formation (green arrow) and elimination (red arrow) during training. Segments are imaged again following 4 days rest (right) to identify boutons formed, eliminated, and maintained during rest, and training-associated boutons that are stabilized (light green) or not stabilized (pink). Extraneous fluorescence structures

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masked and image slightly smoothed for illustration purposes only. (**B**): Bouton formation and elimination during training (black) and during rest (grey). Bouton elimination was significantly elevated during training, p=0.001, n=58 segments, Mann-Whitney U test. 314 baseline boutons, 40 formed during training, 42 formed during rest, 64 eliminated during training, 23 eliminated during rest. Data acquired from 6 mice. Statistics performed across axonal segments. (**C**): Pre-existing bouton survival curves across imaging days. Dotted line depicts baseline bouton survival, reproduced from (Grillo et al., 2013). (**D**): The fraction of boutons maintained during the rest period, measured for pre-existing boutons (present on day 0) that were still present on day 4 following training (black) and boutons formed during training (training-associated boutons, grey). $p=10^{-6}$, Mann-Whitney U test.

Figure 3 - Increased stability of axonal boutons during training in *MECP2*-duplication mice. (**A**): Bouton formation during training (training-associated boutons) and during rest in *MECP2*-duplication mice and WT littermates. (**B**): Pre-existing bouton elimination during training and during rest in each genotype. (**C**): Pre-existing bouton survival curves across imaging. (**D**): Training-associated bouton stabilization rate – the number of boutons formed during training and still present after 4 days of post-training rest is not significantly different across genotypes. Data are plotted as percentage of boutons formed during training. Statistics in A-C, linear mixed-effects models ANOVA; in D, Mann-Whitney U test.

Figure 4 - Sketch of structural plasticity phenotypes in dendrites and axonal projections in area M1 of *MECP2*-duplication and WT mice. A highly simplified diagram of the layer 1 motor cortex circuit, including major local connections, inputs, and outputs. The imaged input projection is shown on the right in bold and represents axonal projections to layer 1 from L5 pyramidal neurons in somatosensory, premotor, and contralateral motor cortex. In WT mice (navy blue), spine formation increases in L5B neuron apical dendrites during motor training, while bouton elimination increases in L5 axonal projections. In *MECP2*-duplication mice (orange), spine formation/stabilization increases even more than WT during training, while bouton elimination is unchanged. See text for detail.

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REFERENCES

Antar LN, Li C, Zhang H, Carroll RC, Bassell GJ (2006) Local functions for FMRP in axon growth cone motility and activity-dependent regulation of filopodia and spine synapses. Mol Cell Neurosci 32:37–48.

Antonova I, Arancio O, Trillat C, Wang HG, Zablow L, Udo H, Kandel ER, Hawkins RD (2001) Rapid increase in clusters of presynaptic proteins at onset of long-lasting potentiation. Science 294:1547–1550.

Ash RT, Buffington SA, Park J, Costa-Mattioli M, Zoghbi HY, Smirnakis SM (2017) Excessive ERK-dependent synaptic clustering with enhanced motor learning in the MECP2 duplication syndrome mouse model of autism. bioRxiv.

Becker N, Wierenga CJ, Fonseca R, Bonhoeffer T, Nägerl UV (2008) LTD Induction Causes Morphological Changes of Presynaptic Boutons and Reduces Their Contacts with Spines. Neuron 60:590–597.

Belichenko PV, Wright EE, Belichenko NP, Masliah E, Li HH, Mobley WC, Francke U (2009) Widespread changes in dendritic and axonal morphology in Mecp2-mutant mouse models of Rett syndrome: Evidence for disruption of neuronal networks. J Comp Neurol 514:240–258.

Blundell J, Kaeser PS, Südhof TC, Powell CM (2010) RIM1 and Interacting Proteins Involved in Presynaptic Plasticity Mediate Prepulse Inhibition and Additional Behaviors Linked to Schizophrenia. J Neurosci 30:5326–5333.

Bourne JN, Chirillo MA, Harris KM (2013) Presynaptic ultrastructural plasticity along CA3->CA1 axons during long-term potentiation in mature hippocampus. J Comp Neurol 521:3898–3912.

Buitrago MM, Schulz JB, Dichgans J, Luft AR (2004) Short and long-term motor skill learning in an accelerated rotarod training paradigm. Neurobiol Learn Mem 81:211–216.

Carrillo J, Cheng S-Y, Ko KW, Jones TA, Nishiyama H (2013) The long-term structural

eNeuro Accepted Manuscript

563 plasticity of cerebellar parallel fiber axons and its modulation by motor learning. J
564 Neurosci 33:8301–8307.

565 Chen J, Yu S, Fu Y, Li X (2014) Synaptic proteins and receptors defects in autism
566 spectrum disorders. Front Cell Neurosci 8:276.

567 Chklovskii DB, Mel BW, Svoboda K (2004) Cortical rewiring and information storage.
568 Nature 431:782–788.

569 Cho RH, Segawa S, Okamoto K, Mizuno A, Kaneko T (2004) Intracellularly labeled
570 pyramidal neurons in the cortical areas projecting to the spinal cord: II. Intra- and
571 juxta-columnar projection of pyramidal neurons to corticospinal neurons. Neurosci
572 Res 50:395–410.

573 Colechio EM, Alloway KD (2009) Differential topography of the bilateral cortical
574 projections to the whisker and forepaw regions in rat motor cortex. Brain Struct
575 Funct 213:423–439.

576 Collingridge GL, Peineau S, Howland JG, Wang YT (2010) Long-term depression in the
577 CNS. Nat Rev Neurosci 11:459–473.

578 Collins AL, Levenson JM, Vilaythong AP, Richman R, Armstrong DL, Noebels JL,
579 Sweatt D, J, Zoghbi HY (2004) Mild overexpression of MeCP2 causes a
580 progressive neurological disorder in mice. Hum Mol Genet 13:2679–2689.

581 D'Antoni S, Spatuzza M, Bonaccorso CM, Musumeci SA, Ciranna L, Nicoletti F, Huber
582 KM, Catania MV (2014) Dysregulation of group-I metabotropic glutamate (mGlu)
583 receptor mediated signalling in disorders associated with Intellectual Disability and
584 Autism. Neurosci Biobehav Rev 46:228–241.

585 De Paola V, Holtmaat A, Knott G, Song S, Wilbrecht L, Caroni P, Svoboda K (2006) Cell
586 type-specific structural plasticity of axonal branches and boutons in the adult
587 neocortex. Neuron 49:861–875.

588 Degano AL, Pasterkamp RJ, Ronnett GV (2009) MeCP2 deficiency disrupts axonal
589 guidance, fasciculation, and targeting by altering Semaphorin 3F function. Mol Cell

eNeuro Accepted Manuscript

590 Neurosci 42:243–254.

591 Deng PY, Rotman Z, Blundon JA, Cho Y, Cui J, Cavalli V, Zakharenko SS, Klyachko VA
592 (2013) FMRP Regulates Neurotransmitter Release and Synaptic Information
593 Transmission by Modulating Action Potential Duration via BK Channels. Neuron
594 77:696–711.

595 Druckmann S, Feng L, Lee B, Yook C, Zhao T, Magee JC, Kim J (2014) Structured
596 Synaptic Connectivity between Hippocampal Regions. Neuron 81:629–640.

597 Federmeier KD, Kleim JA, Greenough WT (2002) Learning-induced multiple synapse
598 formation in rat cerebellar cortex. Neurosci Lett 332:180–184.

599 Feng G, Mellor RH, Bernstein M, Keller-Peck C, Nguyen QT, Wallace M, Nerbonne JM,
600 Lichtman JW, Sanes JR (2000) Imaging neuronal subsets in transgenic mice
601 expressing multiple spectral variants of GFP. Neuron 28:41–51.

602 Garcia-Junco-Clemente P, Golshani P (2014) PTEN: A master regulator of neuronal
603 structure, function, and plasticity. Commun Integr Biol 7.

604 Geinisman Y, Berry RW, Disterhoft JF, Power JM, Van der Zee E (2001) Associative
605 learning elicits the formation of multiple-synapse boutons. J Neurosci 21:5568–
606 5573.

607 Grillo FW, Song S, Teles-Grilo Ruivo LM, Huang L, Gao GG, Knott GW, Maco B,
608 Ferretti V, Thompson D, Little GE, De Paola V (2013) Increased axonal bouton
609 dynamics in the aging mouse cortex. Proc Natl Acad Sci 110:1–10.

610 Holtmaat A, Bonhoeffer T, Chow DK, Chuckowree J, De Paola V, Hofer SB, Hübener M,
611 Keck T, Knott G, Lee W-CA, Mostany R, Mrsic-Flogel TD, Nedivi E, Portera-Cailliau
612 C, Svoboda K, Trachtenberg JT, Wilbrecht L (2009) Long-term, high-resolution
613 imaging in the mouse neocortex through a chronic cranial window. Nat Protoc
614 4:1128–1144.

615 Hooks BM, Mao T, Gutnisky DA, Yamawaki N, Svoboda K, Shepherd GMG (2013)
616 Organization of Cortical and Thalamic Input to Pyramidal Neurons in Mouse Motor

eNeuro Accepted Manuscript

617 Cortex. J Neurosci 33:748–760.

618 Jiang M, Ash RT, Baker SA, Suter B, Ferguson A, Park J, Rudy J, Torsky SP, Chao H-
619 T, Zoghbi HY, Smirnakis SM (2013) Dendritic arborization and spine dynamics are
620 abnormal in the mouse model of MECP2 duplication syndrome. J Neurosci
621 33:19518–19533.

622 Johnson CM, Peckler H, Tai L-H, Wilbrecht L (2016) Rule learning enhances structural
623 plasticity of long-range axons in frontal cortex. Nat Commun 7:10785.

624 Kim J, Zhao T, Petralia RS, Yu Y, Peng H, Myers E, Magee JC (2011) mGRASP
625 enables mapping mammalian synaptic connectivity with light microscopy. Nat
626 Methods 9:96–102.

627 Knott GW, Holtmaat A, Wilbrecht L, Welker E, Svoboda K (2006) Spine growth
628 precedes synapse formation in the adult neocortex in vivo. Nat Neurosci 9:1117–
629 1124.

630 Lee KJ, Park IS, Kim H, Greenough WT, Pak DTS, Rhyu IJ (2013) Motor Skill Training
631 Induces Coordinated Strengthening and Weakening between Neighboring
632 Synapses. J Neurosci 33:9794–9799.

633 Majewska AK, Newton JR, Sur M (2006) Remodeling of Synaptic Structure in Sensory
634 Cortical Areas In Vivo. J Neurosci 26.

635 Mao T, Kusefoglu D, Hooks BM, Huber D, Petreanu L, Svoboda K (2011) Long-Range
636 Neuronal Circuits Underlying the Interaction between Sensory and Motor Cortex.
637 Neuron 72:111–123.

638 Mostany R, Anstey JE, Crump KL, Maco B, Knott G, Portera-Cailliau C (2013) Altered
639 Synaptic Dynamics during Normal Brain Aging. J Neurosci 33:4094–4104.

640 Mostany R, Portera-Cailliau C (2008) A Craniotomy Surgery Procedure for Chronic
641 Brain Imaging. J Vis Exp:e680–e680.

642 Nagerl UV, Kostinger G, Anderson JC, Martin KA, Bonhoeffer T (2007) Protracted

eNeuro Accepted Manuscript

643 synaptogenesis after activity-dependent spinogenesis in hippocampal neurons. J
644 Neurosci 27:8149–8156.

645 Nicholson DA, Geinisman Y (2009) Axospinous synaptic subtype-specific differences in
646 structure, size, ionotropic receptor expression, and connectivity in apical dendritic
647 regions of rat hippocampal CA1 pyramidal neurons. J Comp Neurol 512:399–418.

648 Ramocki MB, Tavyev YJ, Peters SU (2010) The MECP2 duplication syndrome. Am J
649 Med Genet Part A 152:1079–1088.

650 Rothwell PE, Fuccillo MV, Maxeiner S, Hayton SJ, Gokce O, Lim BK, Fowler SC,
651 Malenka RC, Südhof TC (2014) Autism-associated neuroligin-3 mutations
652 commonly impair striatal circuits to boost repetitive behaviors. Cell 158:198–212.

653 Shepherd GM, Harris KM (1998) Three-dimensional structure and composition of CA3 -
654 CA1 axons in rat hippocampal slices: implications for presynaptic connectivity and
655 compartmentalization. J Neurosci 18:8300–8310.

656 Stettler DD, Yamahachi H, Li W, Denk W, Gilbert CD (2006) Axons and synaptic
657 boutons are highly dynamic in adult visual cortex. Neuron 49:877–887.

658 Tennant KA, Adkins DL, Donlan NA, Asay AL, Thomas N, Kleim JA, Jones TA (2011)
659 The organization of the forelimb representation of the C57BL/6 mouse motor cortex
660 as defined by intracortical microstimulation and cytoarchitecture. Cereb Cortex
661 21:865–876.

662 Toni N, Buchs P, Nikonenko I, Bron CR, Muller D (1999) LTP promotes formation of
663 multiple spine synapses between a single axon terminal and a dendrite. Nature
664 402:421–425.

665 Treffert DA (2014) Savant syndrome: Realities, myths and misconceptions. J Autism
666 Dev Disord 44:564–571.

667 White EL, Weinfeld E, Lev DL (2004) Quantitative analysis of synaptic distribution along
668 thalamocortical axons in adult mouse barrels. J Comp Neurol 479:56–69.

eNeuro Accepted Manuscript

669 Wiegert JS, Oertner TG (2013) Long-term depression triggers the selective elimination
670 of weakly integrated synapses. Proc Natl Acad Sci U S A 110:E4510–E4519.

671 Woolley CS, Wenzel HJ, Schwartzkroin PA (1996) Estradiol increases the frequency of
672 multiple synapse boutons in the hippocampal CA1 region of the adult female rat. J
673 Comp Neurol 373:108–117.

674 Xu T, Yu X, Perlik AJ, Tobin WF, Zweig JA, Tennant K, Jones T, Zuo Y (2009) Rapid
675 formation and selective stabilization of synapses for enduring motor memories.
676 Nature 462:915–919.

677 Yang G, Pan F, Gan WB (2009) Stably maintained dendritic spines are associated with
678 lifelong memories. Nature 462:920–924.

679 Yankova M, Hart S, Woolley CS (2001) Estrogen increases synaptic connectivity
680 between single presynaptic inputs and multiple postsynaptic CA1 pyramidal cells: a
681 serial electron-microscopic study. Proc Natl Acad Sci U S A 98:3525–3530.

682

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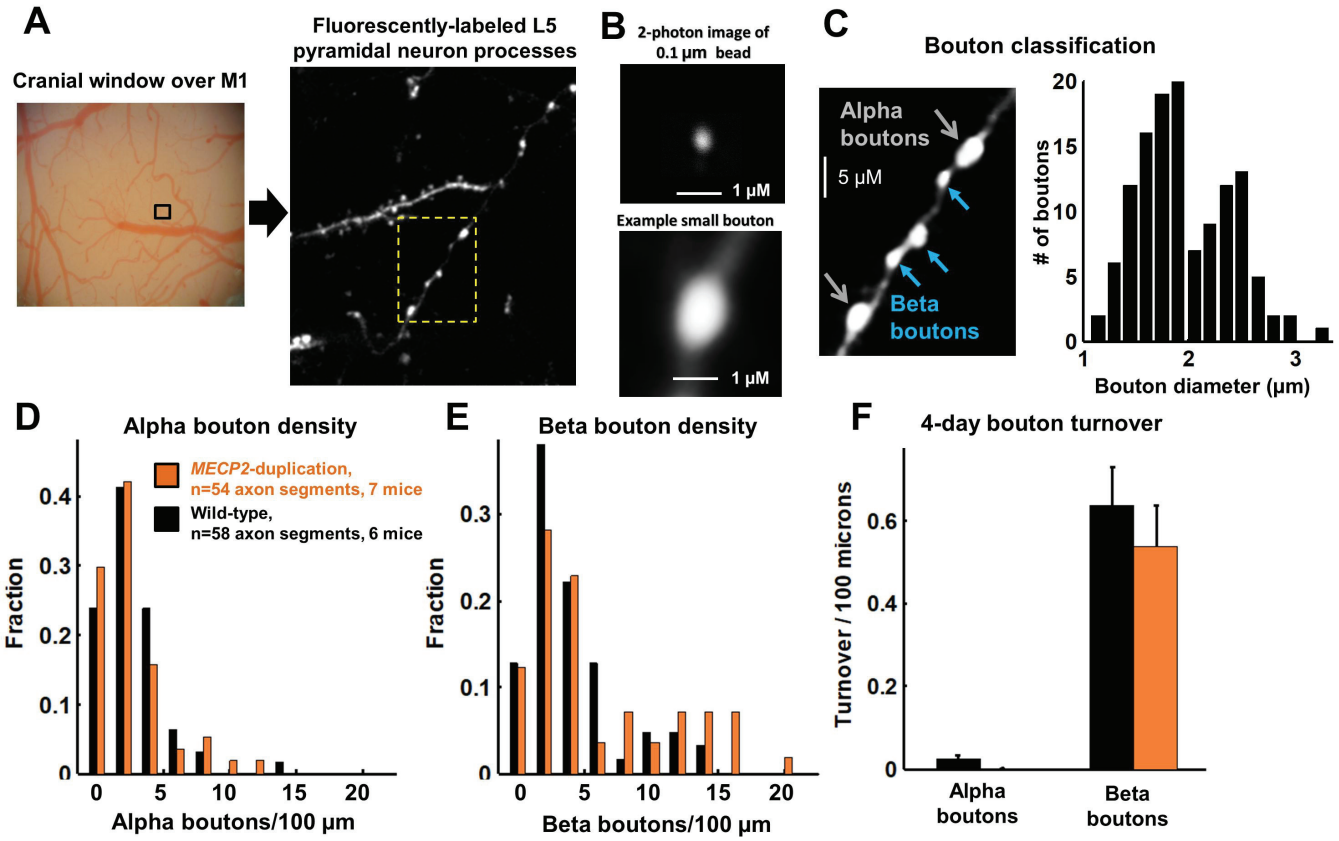

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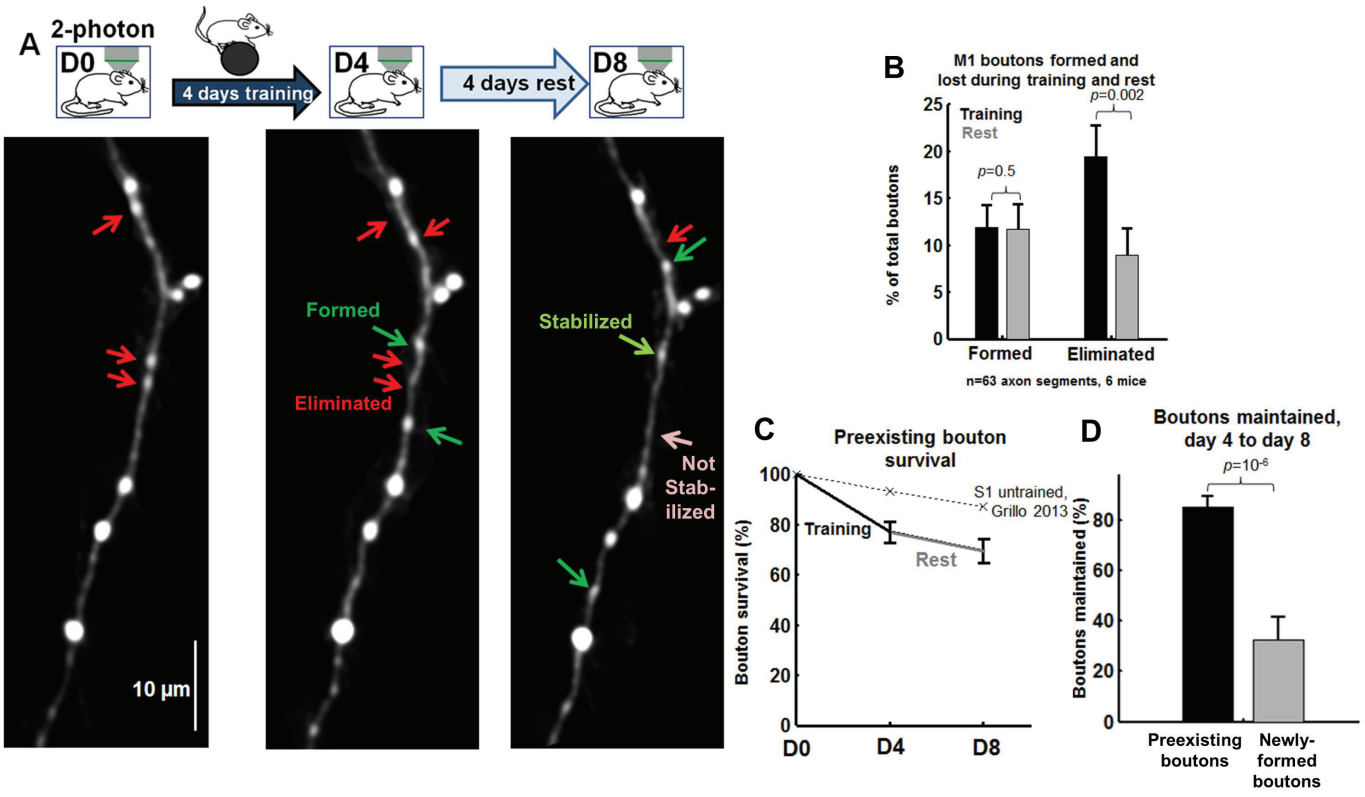

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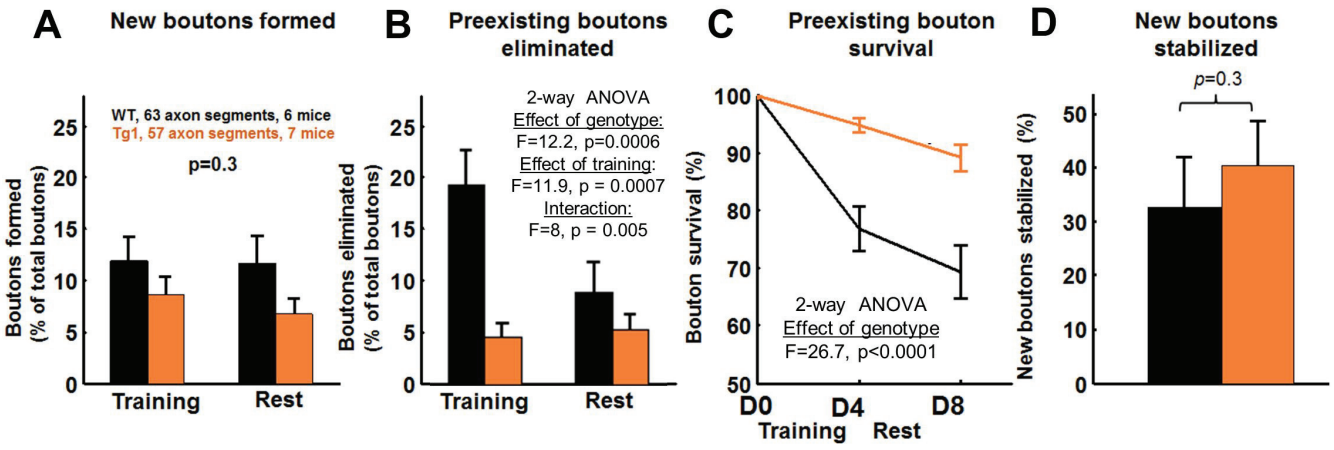

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