

1 **MEF2C hypofunction in neuronal and neuroimmune populations cooperate to produce MEF2C**  
2 **haploinsufficiency syndrome-like behaviors in mice**

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1 **Summary:**

2 Microdeletions of the *MEF2C* gene are linked to a syndromic form of autism termed *MEF2C*  
3 haploinsufficiency syndrome (MCHS). Here, we show that MCHS-associated missense mutations cluster  
4 in the conserved DNA binding domain and disrupt *MEF2C* DNA binding. DNA binding-deficient global  
5 *Mef2c* heterozygous mice (*Mef2c*-Het) display numerous MCHS-like behaviors, including autism-related  
6 behaviors, as well as deficits in cortical excitatory synaptic transmission. We find that hundreds of genes  
7 are dysregulated in *Mef2c*-Het cortex, including significant enrichments of autism risk and excitatory  
8 neuron genes. In addition, we observe an enrichment of upregulated microglial genes, but not due to  
9 neuroinflammation in the *Mef2c*-Het cortex. Importantly, conditional *Mef2c* heterozygosity in forebrain  
10 excitatory neurons reproduces a subset of the *Mef2c*-Het phenotypes, while conditional *Mef2c*  
11 heterozygosity in microglia reproduces social deficits and repetitive behavior. Together our findings  
12 suggest that *MEF2C* regulates typical brain development and function through multiple cell types,  
13 including excitatory neuronal and neuroimmune populations.

14

15 **Keywords:** *Mef2c*, mouse, autism, neuron, microglia

1 **Introduction:**

2 Myocyte Enhancer Factor 2 (MEF2) proteins are members of the MADS family of transcription  
3 factors that regulate gene expression during development and adulthood. In the brain, MEF2C is  
4 important for neuronal differentiation and synapse development (Assali et al., 2019). MEF2 proteins  
5 regulate numerous genes associated with synapse formation and function as well as multiple genes  
6 linked to neurodevelopmental disorders, including autism spectrum disorder (ASD) (Flavell et al., 2008;  
7 Harrington et al., 2016; Morrow et al., 2008). Constitutively-active MEF2C can promote glutamatergic  
8 synapse elimination, a process requiring the RNA-binding function of the Fragile X Mental Retardation  
9 protein (FMRP) and several other factors (Flavell et al., 2006; Pfeiffer et al., 2010; Tsai et al., 2012; Zang  
10 et al., 2013). Conditional knockout of *Mef2c* in neuronal populations within the mouse brain produces a  
11 myriad of severe behavioral and synaptic phenotypes, which emphasizes the importance of this gene in  
12 healthy brain development (Adachi et al., 2015; Barbosa et al., 2008; Harrington et al., 2016; Li et al.,  
13 2008; Rajkovich et al., 2017).

14 MEF2C in the developing and mature brain is also expressed in microglia (Deczkowska et al.,  
15 2017; Gosselin et al., 2017; Zhang et al., 2014a) – a population of macrophage-like cells throughout the  
16 brain that regulate synapse formation and pruning during early brain development (Paolicelli et al., 2011;  
17 Schafer et al., 2012; Zhan et al., 2014). Microglia influence a number of brain functions, including synapse  
18 elimination, synapse formation, fasciculation of the corpus callosum, survival of oligodendrocyte  
19 precursor cells, and phagocytosis of other brain cells (Hagemeyer et al., 2017; Parkhurst et al., 2013;  
20 Pont-Lezica et al., 2014; Schafer et al., 2012; Shigemoto-Mogami et al., 2014; Sierra et al., 2010; Stevens  
21 et al., 2007). Microglia are recognized as not just responding to infection or injury, but as important  
22 regulators of brain development and function (Li and Barres, 2018). In addition, microglial dysfunction  
23 has been hypothesized to play important roles in disease pathology for other neurodevelopmental  
24 disorders, including Rett Syndrome (Derecki et al., 2012; Horiuchi et al., 2017; Schafer et al., 2016; Wang  
25 et al., 2015).

26 Microdeletions on chromosome 5q14.3 that include the *MEF2C* gene or point mutations within  
27 the protein-coding region of *MEF2C* are linked to a recently described neurodevelopmental disorder,  
28 termed *MEF2C* Haploinsufficiency Syndrome (MCHS) (Berland and Houge, 2010; Bienvenu et al., 2013;

1 Engels et al., 2009; Le Meur et al., 2010; Mikhail et al., 2011; Novara et al., 2010; Paciorkowski et al.,  
2 2013; Tonk et al., 2011; Vrekar et al., 2017; Zweier et al., 2010; Zweier and Rauch, 2012). Common  
3 symptoms of MCHS include autism spectrum disorder (ASD), absence of speech, stereotypical  
4 behaviors, hyperactivity, intellectual disability, hypotonia, motor abnormalities, high pain tolerance, sleep  
5 disturbances, and epilepsy, and individuals with *MEF2C* point mutations typically present with fewer  
6 and/or milder symptoms (Berland and Houge, 2010; Bienvenu et al., 2013; Engels et al., 2009; Le Meur  
7 et al., 2010; Mikhail et al., 2011; Novara et al., 2010; Paciorkowski et al., 2013; Tonk et al., 2011; Vrekar  
8 et al., 2017; Zweier et al., 2010; Zweier and Rauch, 2012). Due to the abundance of neurological  
9 symptoms and neuronal-enriched expression of *MEF2C*, *MEF2C* haploinsufficiency in neurons is  
10 presumed to underlie most, if not all, of the MCHS symptoms. Interestingly, single-cell genomic profiling  
11 from cortical tissue of patients with idiopathic autism revealed that upper-layer excitatory neurons and  
12 microglia are preferentially affected in autism (Velmeshev et al., 2019), and since both neurons and  
13 microglia express *MEF2C*, we sought to explore the possible cell type-specific effects of *MEF2C*  
14 hypofunction in MCHS-related behaviors in a construct-valid mouse model of human MCHS.

15

## 16 **Methods and Materials:**

### 17 **Patients**

18 Patients with developmental delay and a significant variant in the *MEF2C* gene were selected for this  
19 study. These patients were seen for clinical genetics evaluations and data from these visits were gathered  
20 from records review. Internal informed consent to publish data was obtained for each subject.

21

### 22 **Animals**

23 Mice (*Mus musculus*) were group housed (2-5 mice/cage; unless specified) with same-sex littermates  
24 with access to food and water *ad libitum* on a 12-hour reverse light-dark cycle. *Mef2c*<sup>+/-</sup> (*Mef2c*-Het) mice  
25 were initially generated by crossing *Mef2c*<sup>fl/fl</sup> (RRID:MGI:3719006) (Arnold et al., 2007) mice with *Prm1*-  
26 *Cre* (Jackson Laboratory #003328) to induce germline recombination of *Mef2c*. *Mef2c*<sup>+/-</sup>; *Prm1*-*Cre* were  
27 backcrossed with C57BL/6J to remove *Prm1*-*Cre*, and test mice were generated from *Mef2c*<sup>+/-</sup> mice (male  
28 and female) crossed with C57BL/6J mice. *Mef2c* conditional heterozygous mice were generated by

1 crossing *Mef2c* floxed mice with cell type-selective Cre<sup>+</sup>-expressing transgenic mice (*Emx1-Cre* (Iwasato  
2 et al., 2008)), *PV-Cre* (Jackson Laboratory #017320), or *Cx3Cr1<sup>creER/creER</sup>* (Jackson Laboratory #021160  
3 (Parkhurst et al., 2013)) to generate *Mef2c<sup>fl/+</sup>; Cre<sup>+</sup>* conditional heterozygous (*Mef2c* cHet) mice that were  
4 compared to their Cre-negative or flox-negative littermates (Control). Experimenters were blinded to the  
5 mouse genotype during data acquisition and analysis. Experiments were independently replicated, and  
6 the total number of animals/cells were reported in the representative figures. All procedures were  
7 conducted in accordance with the Medical University of South Carolina Institutional Animal Care and Use  
8 Committee (IACUC) and NIH guidelines.

9

10 *Detailed Materials and Methods can be found within the supplemental information.*

11

## 12 **Results:**

### 13 **Patient *MEF2C* missense mutations cluster in DNA binding and dimerization domains and disrupt**

#### 14 **DNA binding**

15 Deletions or mutations in *MEF2C* are assumed to create loss-of-function alleles that cause the  
16 symptoms of MCHS (Berland and Houge, 2010; Bienvenu et al., 2013; Engels et al., 2009; Le Meur et  
17 al., 2010; Mikhail et al., 2011; Novara et al., 2010; Paciorkowski et al., 2013; Tonk et al., 2011; Vrekar et  
18 al., 2017; Zweier et al., 2010; Zweier and Rauch, 2012). Given that microdeletions of 5q14.3 often include  
19 additional genes beyond *MEF2C*, we sought to identify individuals with mutations within the *MEF2C*  
20 protein-coding region, including an intragenic duplication (*i.e.* p.D40\_C41dup) and two missense variants  
21 (*i.e.* p.K30N and p.I46T) (Table 1). We compared their clinical histories to those associated with two  
22 previously reported missense variants in the *MEF2C* gene (Zweier et al., 2010). All five patients presented  
23 with global developmental delay and seizures. Seizure onset occurred prior to one year of age in four of  
24 the five patients, with the patient carrying the p.I46T variant developing seizures at 21 months. Common  
25 features of these individuals included absence of speech, repetitive movements, hypotonia, varied  
26 abnormalities on brain MRI, and breathing disturbances. High pain tolerance was noted in two of the  
27 patients. There were some minor facial dysmorphism noted, though there did not seem to be a  
28 consistently recognizable gestalt. When a list of additional MCHS mutations was assembled (personal

1 communications), several frameshift and premature stop codon mutations were identified – all of which,  
2 if stable, are predicted to produce a truncated MEF2C protein lacking its C-terminal nuclear localization  
3 sequence. We also noted that all of the *MEF2C* missense (or small duplication) mutations were clustered  
4 within the highly-conserved MADS (DNA binding) or MEF2 (dimerization) domains (Fig. 1A). In a MEF2  
5 response element DNA binding assay, all five of the MADS domain patient mutations caused a loss of  
6 MEF2C DNA binding (Fig. 1B-C, S1A), and they did not appear to interfere with wild-type MEF2C DNA  
7 binding (Fig. S1B), suggesting a loss-of-function phenotype.

8

### 9 ***Mef2c* heterozygous mouse model**

10 To model the genetics of MCHS in mice, we generated a global heterozygous *Mef2c* mutant  
11 mouse lacking exon 2 (*Mef2c*<sup>+/ $\Delta$ Ex2</sup> or *Mef2c*-Het) (Fig. 1D), which encodes a large portion of the  
12 MADS/MEF2 domains. The near full-length MEF2C <sup>$\Delta$ Ex2</sup> protein had no detectable DNA binding affinity  
13 and did not reduce DNA binding affinity of wild-type MEF2C expressed at a similar level (Fig. 1E-F, S1C).  
14 We observed a non-Mendelian frequency of *Mef2c*-Hets, suggesting a partial embryonic lethality  
15 (Extended Data Fig. 1D, \*\*p<0.01, Chi-square test), similar to a previous report (Tu et al., 2017).

16 We examined whether male and female *Mef2c*-Het mice showed behavior phenotypes  
17 reminiscent of MCHS symptoms. Using a three-chamber social interaction (SI) test, we observed that  
18 *Mef2c*-Het mice have a lack of social preference with a novel same-sex mouse (Fig. 2A, \*p<0.05, 2-way  
19 ANOVA). We also found that *Mef2c*-Het male and female pups (P7-P10) produced significantly fewer  
20 ultrasonic vocalization (USV) calls during maternal separation (Fig. 2B, main effect of genotype,  
21 \*\*p<0.01), and young adult *Mef2c*-Het males produced significantly fewer USV calls (Fig. 2C, \*p<0.05)  
22 in the presence of an estrous female, suggesting that *Mef2c*-Hets have deficits in a putative species-  
23 appropriate form of social communication. Male *Mef2c*-Hets were hyperactive in a novel environment  
24 (Fig. 2E, \*\*\*p<0.005) and displayed an increase in jumping (Fig. 2F, \*p<0.05), a repetitive-type motor  
25 behavior; however, young adult *Mef2c*-Hets displayed normal performance on the accelerating rotarod  
26 test of motor coordination (Fig. 2D). In addition, *Mef2c*-Hets showed increased exploration of the open,  
27 unprotected arm of the elevated plus maze (Fig. 2G). High pain tolerance is also frequently reported in  
28 MCHS individuals (Paciorkowski et al., 2013; Zweier et al., 2010). Interestingly, *Mef2c*-Het mice showed

1 a reduction in startle response to electrical foot-shocks (Fig. 2H; 2-way ANOVA, significant interaction  
2 between genotype and shock intensity; \* $p < 0.05$ , \*\* $p < 0.01$ ); however, this might be specific to pain stimuli  
3 (nociception) given that white-noise acoustic startle responses are indistinguishable from controls (Fig.  
4 S2A). Despite a common occurrence of intellectual disability in MCHS, in the *Mef2c*-Hets we failed to  
5 detect any clear learning and memory-related deficits in Pavlovian fear conditioning tests (Fig. S2B-D),  
6 the Barnes maze test for spatial learning and memory (Fig. S2E), and the Y-maze test for spatial working  
7 memory (Fig. S2F). These mice also showed a strong preference for the novel object in the novel object  
8 recognition test (Fig. S2G), and normal sucrose preference in a two-bottle choice test (Fig. S2H). In the  
9 cognitively-demanding operant sucrose self-administration (SA) assay, the *Mef2c*-Hets displayed wild-  
10 type levels of operant learning, operant discrimination (active vs. inactive port), context-related sucrose  
11 seeking after one-week of abstinence, extinction learning and cue-induced reinstatement of sucrose  
12 seeking on the formerly-active port (Fig. 2I-J, S2I-L). Taken together, our findings suggest that, unlike  
13 the conditional knockout of *Mef2c* in *Emx1*-lineage cells (Harrington et al., 2016), the global loss of one  
14 functional copy of *Mef2c* in mice is not sufficient to produce detectable deficits in learning or memory in  
15 the C57BL6/J genetic background.

16

### 17 ***Mef2c*-Het mice display input-selective reductions in cortical excitatory synaptic transmission**

18 Changes in excitatory (E) and/or inhibitory (I) synaptic transmission are associated with numerous  
19 neuropsychiatric disorders, including ASD (Antoine et al., 2019; Garber, 2007; Zoghbi and Bear, 2012).  
20 Conditional knockout of *Mef2c* alters somatosensory cortex (SSCtx) pyramidal neuron E/I synaptic  
21 transmission (Harrington et al., 2016; Rajkovich et al., 2017). In the global *Mef2c*-Hets (p35-p40), gross  
22 structural organization of barrel fields within cortical layer 4 of the SSCtx appeared normal (Fig. 3A). In  
23 SSCtx layer 2/3 pyramidal neurons, we detected no significant differences by genotype for intrinsic  
24 excitability (Fig. S3A), dendritic spine density or spine head diameter of apical or basal dendrites (Fig.  
25 S3B), or GABA-mediated inhibitory synaptic transmission (mIPSCs; Fig. 3B). However, patch-clamp  
26 recordings of layer 2/3 neurons revealed an input-selective deficit in glutamatergic synaptic transmission.  
27 Stimulation of horizontal fibers in layer 2/3 of a neighboring cortical column produced a significant  
28 reduction in the amplitude of evoked excitatory postsynaptic currents (eEPSCs) (Fig. 3C), suggesting a

1 reduction in pre- and/or postsynaptic transmission. Paired-pulse facilitation (PPF) analysis (50 ms  
2 interstimulus interval) of local horizontal inputs revealed a significant increase in PPF ratio (Fig. 3C),  
3 indicating a decrease in presynaptic release probability (Fioravante and Regehr, 2011). These effects  
4 were input-selective given that electrical stimulation of layer 4 (within the same cortical column) produced  
5 eEPSC and PPF responses in layer 2/3 neurons that were indistinguishable from controls (Fig. 3D). To  
6 examine if reductions in AMPA-mediated postsynaptic strength might also contribute to the reduced  
7 horizontal eEPSCs (Fig. 3C), we measured miniature EPSCs (mEPSCs) under conditions where action  
8 potentials are blocked pharmacologically. In layer 2/3 cells from *Mef2c*-Hets, we observed a significant  
9 reduction in mEPSCs amplitude (Fig. 3E), suggesting an overall reduction in AMPA-mediated  
10 postsynaptic strength. Similar to layer 2/3, we also observed a significant reduction of mEPSC amplitude  
11 in SSCtx layer 5 pyramidal neurons of *Mef2c*-Hets (Fig. 3F), suggesting that the reduction in  
12 glutamatergic postsynaptic strength is not limited to a specific cortical layer. Consistent with layer 2/3  
13 pyramidal neurons, we did not observe any differences in dendritic spine density or dendritic spine head  
14 diameter in basal dendrites from layer 5 pyramidal neurons (Fig. S3C). There was no effect of genotype  
15 on layer 5 mEPSC frequency (Fig. 3F), but we observed a significant increase in the layer 2/3 mEPSCs  
16 frequency (Fig. 3E) that was not explained by an increase in dendritic spine density (Fig. S3B) or effects  
17 on presynaptic functions of local inputs (Fig. 3C,D), and might represent a compensatory effect of long-  
18 range connections (Rajkovich et al., 2017).

19

## 20 ***Mef2c*-Het mice display dysregulation of cortical genes associated with ASD risk, excitatory** 21 **neurons and microglia**

22 Using an unbiased RNA-sequencing (RNA-Seq) approach, we examined gene expression from  
23 whole cortex in control and *Mef2c*-Hets (p35-p40), and we identified 490 genes that were significantly  
24 dysregulated (FDR < 0.05; Fig. 4A, S4A; Table S1,S2). We confirmed the differential expression of a  
25 number of interesting *Mef2c*-Het differentially expressed genes (DEGs) that are associated with ASD  
26 risk, microglia, and others by qRT-PCR (Fig. 4D). We also investigated the association of *Mef2c*-Het  
27 DEGs with genetic and genomic data from various brain disorders. We found that the *Mef2c*-Het DEGs,  
28 particularly the downregulated genes, were overrepresented in genes associated with ASD risk and



1 FMRP binding (Fig. 4B; Table S1; Fig. 4D). We also assessed enrichment for *Mef2c*-Het DEGs in genes  
2 that are dysregulated in a meta-analysis of transcriptomic data across neuropsychiatric disorders (Gandal  
3 et al., 2018). Interestingly, *Mef2c*-Het DEGs, particularly the downregulated genes, were significantly  
4 enriched for a PsychENCODE excitatory neuron module of genes that are downregulated in ASD (versus  
5 other neuropsychiatric disorders) brains (geneM1; Fig. 4C; Table S2). We also observed significant  
6 enrichment of *Mef2c*-Het DEGs in PsychENCODE gene module 8 (geneM8; Fig. 4C; Table S2), which is  
7 an excitatory neuron module of genes downregulated in both ASD and schizophrenia and enriched for  
8 Bipolar Disorder (BPD) genetic variants. *Mef2c*-Het DEGs, particularly the upregulated genes, were  
9 enriched in PsychENCODE module 6, which is a microglia module of genes upregulated in ASD, but  
10 downregulated in SCZ and BPD (geneM6; Fig. 4C; Table S2). Using single-cell RNA-seq data from  
11 mouse cortex (Saunders et al., 2018), we observed that *Mef2c*-Het DEGs were strongly enriched for  
12 cortical excitatory neuron genes and microglia genes (Fig. S4B; Table S2), further supporting the  
13 importance of MEF2C in regulating gene expression in the two key brain populations where MEF2C  
14 expression is most highly expressed.

15 Gene ontology analysis of the *Mef2c*-Het DEGs revealed significant enrichment of microglia  
16 proliferation genes, cell metabolism genes, and genes in a microglia subpopulation in the developing  
17 brain that is restricted to unmyelinated axon tracts (Fig. S5). Since *Mef2c*-Hets showed significant  
18 dysregulation of microglial genes (Fig. 4C,D), and MEF2C is expressed in microglia in the developing  
19 and mature brain (Fig. S5) (Deczkowska et al., 2017; Gosselin et al., 2017; Zhang et al., 2014a), we  
20 analyzed the *Mef2c*-Het brain for possible upregulation of the microglia cell-type and neuroimmune  
21 activation marker, ionized calcium-binding adapter molecule 1 (*Iba1*) (Ito et al., 1998; Ito et al., 2001). In  
22 both the cortex and hippocampus, we observed a significant increase in *Iba1* expression (Fig. 5A-C,E;  
23 \*\*\*\* $p < 0.0001$ , K-S test) without a change in the density of microglia (Fig. 5D,F), suggesting possible  
24 microglial activation in the *Mef2c*-Het brain. This increase in *Iba1* was present without an obvious change  
25 in microglial cell morphology or microglial cell soma volume (Figs. 5A,B,G). In addition, in the *Mef2c*-Het  
26 cortex, we observed no changes in classic- and alternative-pathway pro-inflammatory genes, including  
27 IL-1B, TNF $\alpha$ , CD68, IFN $\gamma$ , and several others (Fig. 5G). However, we did note a significant increase in  
28 the expression of several complement-related genes linked previously to synaptic pruning and/or ASD

1 risk, including *C1qb*, *C1qc* and *C4b* (Fig. 4D, (Bialas and Stevens, 2013; Odell et al., 2005; Schafer et  
2 al., 2012; Sekar et al., 2016; Stevens et al., 2007). Moreover, we observed significant enrichments of  
3 upregulated *Mef2c*-Het DEGs in scRNA-seq gene clusters associated with embryonic-like microglia,  
4 postnatal immature microglia, and homeostatic microglia (Fig. 5H). Taken together, these results reveal  
5 that the reduction of MEF2C levels has significant impacts on microglia gene expression programs.

6

## 7 **MEF2C contributes to neurotypical behaviors through key roles in forebrain excitatory neurons** 8 **and microglia**

9 In the developing and mature mouse brain, MEF2C is expressed in several neuronal cell types,  
10 including cortical excitatory pyramidal cells and Parvalbumin-positive GABAergic inhibitory neurons, and  
11 in microglia (Barbosa et al., 2008; Harrington et al., 2016; Kamath and Chen, 2018; Mayer et al., 2018;  
12 Zhang et al., 2014b). Since the *Mef2c*-Het mouse cortex showed robust changes in both excitatory  
13 neurons and microglia gene expression (Figs. 4, S4), we generated cell type-specific conditional *Mef2c*  
14 heterozygous mice to explore the contribution of forebrain excitatory neurons versus microglia for the  
15 development of MCHS-like phenotypes. We first generated mice heterozygous for *Mef2c* in *Emx1*-lineage  
16 cells (*Mef2c*-cHet<sup>*Emx1-cre*</sup>) (Iwasato et al., 2008), which represents ~85% of forebrain excitatory neurons  
17 throughout the cortex and hippocampus. Similar to the global *Mef2c*-Hets, the *Mef2c*-cHet<sup>*Emx1-cre*</sup> mice  
18 displayed altered anxiety-like behavior and male-selective increases in locomotion and repetitive jumping  
19 (Fig. 6A-C), but they showed no changes in social behavior or shock sensitivity (Fig. 6D, S6A).  
20 Interestingly, similar to global *Mef2c*-Hets (Fig. 3E) and *Mef2c* cKO<sup>*Emx1-cre*</sup> mice (Harrington et al., 2016),  
21 we observed a reduction of mEPSC amplitude in layer 2/3 pyramidal neurons from *Mef2c*-cHet<sup>*Emx1-cre*</sup>  
22 mice (Fig. S6D), suggesting that the deficit in cortical glutamatergic synaptic transmission in layer 2/3 of  
23 the global *Mef2c*-Hets is due to MEF2C's function in excitatory forebrain neurons. In addition, mice  
24 heterozygous for *Mef2c* in PV-positive GABAergic interneurons (*Mef2c*-cHet<sup>*PV-cre*</sup>) – a population of  
25 predominantly GABAergic interneurons with high MEF2C expression – also showed male-selective  
26 increases in locomotion (Fig. 6F), but these mice did not develop changes in repetitive jumping, anxiety-  
27 like behavior, social interaction, or shock sensitivity (Figs. 6E,G-H, S6B). These findings suggest that  
28 *Emx1*-lineage excitatory forebrain neurons, and to a much lesser extent PV-expressing neurons,

1 contribute to the development of some, but not all, of the behavior phenotypes observed in the global  
2 *Mef2c*-Hets.

3 To explore a possible contribution of MEF2C in microglia to MCHS-like behaviors, we generated  
4 mice heterozygous for *Mef2c* selectively in microglia (*Mef2c*-cHet<sup>Cx3cr1-cre</sup>). The conditional mutant mice  
5 displayed social impairments in the 3-chamber social interaction test (Fig. 6L, \*p<0.05, two-way ANOVA),  
6 similar to the *Mef2c*-Het mice. In addition, *Mef2c*-cHet<sup>Cx3cr1-cre</sup> mice showed a significant increase in male-  
7 specific repetitive jumping (Fig. 6K, \*p<0.05, Welch's t-test), but with no discernable effects on exploratory  
8 activity (Fig. 6J), anxiety-like behavior or shock sensitivity (Fig. 6I, S6C). Taken together, our  
9 observations suggest that *Mef2c* haploinsufficiency in early postnatal microglial cells is sufficient to  
10 produce autism-related behaviors, and that the majority of MCHS-like phenotypes in the global *Mef2c*-  
11 Hets are a consequence of MEF2C hypofunction in both neurons and microglia.

12

### 13 **Discussion**

14 We report here three new *MEF2C* mutations identified in individuals with MCHS-related  
15 symptoms, and these *MEF2C* missense or small duplication mutations disrupted MEF2C DNA binding.  
16 Interestingly, all of the known MCHS missense or duplication mutations cluster within the highly-  
17 conserved MADS and MEF2 domains (Fig. 1A) that mediate the DNA binding and dimerization functions  
18 of MEF2C (McKinsey et al., 2002). We also found that global, DNA binding-deficient *Mef2c* heterozygous  
19 mice display numerous behavioral phenotypes reminiscent of MCHS, including social interaction deficits,  
20 ultrasonic vocalization deficits, motor hyperactivity, repetitive behavior, anxiety-related behavior and  
21 reduced sensitivity to a painful stimulus (footshock). The *Mef2c*-Hets also possessed input-selective pre-  
22 and postsynaptic deficits in glutamatergic excitatory synaptic transmission in the somatosensory cortex.  
23 Gene expression analysis of cortical tissue from *Mef2c*-Hets revealed significant enrichment of genes  
24 linked to ASD risk, excitatory neurons and microglia, which is notable considering the enrichment of  
25 dysregulated genes linked to cortical excitatory neurons and microglia in brains of individuals with  
26 idiopathic ASD (Velmeshev et al., 2019). Conditional *Mef2c* heterozygous mice in *Emx1*-lineage cells,  
27 which represent predominantly forebrain excitatory neurons, reproduced several of the global *Mef2c*-Het  
28 behaviors and cortical synaptic phenotypes. Consistent with the dysregulation of microglial genes in

1 *Mef2c*-Het mice, early postnatal conditional *Mef2c* heterozygosity in *Cx3cr1*-lineage cells, which in the  
2 brain are almost exclusively microglia (Hoogland et al., 2015; Ito et al., 1998; Ito et al., 2001), produced  
3 offspring with social deficits and increased repetitive behavior. Our findings support the emerging view  
4 that microglial dysfunction in the developing brain can contribute to the development of ASD symptoms.

5 While our findings reveal a key role for MEF2C in both neurons and microglia for neurotypical  
6 behaviors in mice, there are a number of new questions raised by these findings. For example, why do  
7 we see male-selective effects of *Mef2c* heterozygosity on hyperactivity and/or jumping behavior in the  
8 *Mef2c*-Het and *Mef2c*-cHet mice (Figs. 2E,F; 6B,C,F,K)? This suggests an interaction between sex-  
9 based mechanisms of development and MEF2C-dependent transcription during development, and  
10 indeed, numerous studies have demonstrated that both neuron and microglia functions can be  
11 differentially regulated in males and females (Wright-Jin and Gutmann, 2019). It is also interesting to note  
12 that *Mef2c*-Het DEGs linked to excitatory neurons show a preferential downregulation, whereas *Mef2c*-  
13 Het DEGs linked to microglia display a preferential upregulation. MEF2C is reported to function as both  
14 a transcriptional activator and a repressor, and there are cell type-specific signaling mechanisms that  
15 regulate MEF2C activity (Harrington et al., 2016; Kang et al., 2006; Lyons et al., 2012). Determining  
16 whether or how MEF2C regulates cell type-specific gene expression as an activator or repressor, or  
17 whether the DEGs are an indirect consequence of MEF2C hypofunction, will be important goals for future  
18 studies.

19 MEF2 proteins can regulate activity-dependent glutamatergic synapse elimination (Flavell et al.,  
20 2006; Pfeiffer et al., 2010; Pulipparacharuvi et al., 2008; Tsai et al., 2012). MEF2C can function in cortical  
21 pyramidal neurons as a cell-autonomous transcriptional repressor to regulate dendritic spine density,  
22 synapse number and AMPA-mediated postsynaptic strength (Harrington et al., 2016; Rajkovich et al.,  
23 2017). Conditional deletion of both *Mef2c* gene copies in forebrain excitatory neurons produces mice with  
24 dramatic changes in cortical synapse functions, including decreased glutamatergic synaptic  
25 transmission, and numerous alterations in typical mouse behaviors, including learning and memory,  
26 reward-related behavior, motor hyperactivity and repetitive behaviors, and differential gene expression  
27 (Adachi et al., 2015; Barbosa et al., 2008; Harrington et al., 2016; Li et al., 2008). In contrast to these  
28 *Mef2c* brain conditional knockouts, humans with MCHS possess deletions or mutations in a single gene

1 copy throughout the entire body (Berland and Houge, 2010; Bienvenu et al., 2013; Engels et al., 2009;  
2 Le Meur et al., 2010; Mikhail et al., 2011; Novara et al., 2010; Paciorkowski et al., 2013; Tonk et al., 2011;  
3 Vrekar et al., 2017; Zweier et al., 2010; Zweier and Rauch, 2012). Since MCHS symptoms are reported  
4 predominantly from macro- and microdeletions that disrupt *MEF2C* and other neighboring genes, we  
5 sought to identify possible loss-of-function *MEF2C* mutations within its protein coding region to better  
6 understand the relationship between symptoms and *MEF2C*. By comparing multiple new *MEF2C*-related  
7 mutations from individuals with developmental delay and other MCHS-associated symptoms, we  
8 observed that all of the missense mutations resided within the MEF2C DNA binding and dimerization  
9 domains (MADS/MEF2). In addition, there were multiple mutations that produced a premature stop codon  
10 or a frameshift predicted to produce a truncated MEF2C lacking the C-terminal nuclear localization  
11 sequence. One interesting observation was the ~1.3-fold increase in *Mef2c* mRNA levels in the *Mef2c*-  
12 Het mouse cortex (Fig. 4). However, there was a ~50% reduction of the exon 2-containing transcripts  
13 (the Cre-flox edited exon), resulting in ~35% overall reduction in full-length, functional MEF2C (Fig. 4D).  
14 This suggests that MEF2C might negatively regulate its own gene expression, as originally reported in  
15 muscle cells (Wang DZ et al., 2001), and that a relatively modest overall reduction in MEF2C levels is  
16 sufficient to produce numerous changes in gene expression, synaptic development and function, and  
17 neurotypical behaviors.

18         Developing and mature microglia play important roles in brain development, including synaptic  
19 phagocytosis (Paolicelli et al., 2011; Schafer et al., 2012). Microglia also mediate synapse patterning,  
20 neurogenesis, myelinogenesis, and cellular phagocytosis (Parkhurst et al., 2013; Sierra et al., 2010; Zhan  
21 et al., 2014). MEF2C is expressed in both human and mouse microglia, and MEF2 proteins regulate  
22 microglia development (Gosselin et al., 2017). Microglia-enriched RNAs are dysregulated in human  
23 cortex from idiopathic ASD brains (Velmeshev et al., 2019) and in the mouse *Mef2c*-Het cortex (Fig. 4,  
24 S4), and we find that *Mef2c* hypofunction in microglia (Cx3cr1-lineage) is sufficient to produce autism-  
25 like behaviors in mice (Fig. 6K,L). Interestingly, despite a strong increase in the *Mef2c*-Het brain of the  
26 microglia cell-type and activation marker, *Iba1*, (Fig. 5) as well as other microglia genes including several  
27 complement genes (e.g. *C1qb*, *C1qc* and *C4b*), osteopontin (*Spp1*), and Cathepsin S (*Ctss*) (Fig. 4C,D),  
28 we failed to detect a clear signature of neuroinflammation (Fig. 5G). Our findings suggest that loss of one

1 *Mef2c* copy does not produce classic microglial “activation”, but rather that microglial development,  
2 function or maturation might be perturbed. Of note, *Mef2c*-Het DEGs showed enrichment for a scRNA-  
3 seq cluster of genes associated with embryonic and immature postnatal microglia, suggesting a possible  
4 delay in microglia maturation in the *Mef2c*-Het mice. Future studies will be important to determine the  
5 precise roles of MEF2C in microglial development and function, and whether *Mef2c* heterozygosity alters  
6 one or more of the numerous reported roles for microglia in brain development.

7 Taken together our findings reveal that MEF2C hypofunction throughout development produces  
8 numerous complex changes in cortical synaptic transmission, gene expression and behaviors  
9 reminiscent of MCHS and ASD. Specifically, the *Mef2c*-Het behaviors are associated with robust, input-  
10 selective deficits in cortical excitatory synaptic transmission, and disruption of excitatory neuronal and  
11 microglial gene expression. Importantly, our cell type-selective manipulations strongly suggest that  
12 MEF2C contributes to neurotypical development through critical roles in multiple cell types, including  
13 forebrain excitatory neurons (Emx1-lineage), PV-positive interneurons and microglia. Understanding the  
14 role of MEF2C in these, and other, cell populations in the body are likely to provide important new insights  
15 into effective treatment strategies for symptoms of MCHS.

16

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25

### 26 **Author Contributions:**

27 A.J.H., C.M.B., A.A., and C.W.C. designed experiments, performed data analysis, and wrote the  
28 manuscript. H.W.M, D.B.E., and S.A.S. collected MCHS patient data. A.J.H., C.M.B., A.A., K.B., and



1 Y.J.C. performed behavior test and analyzed data. S.B. and G.K. analyzed RNA-Seq data. E.T.  
2 performed electrophysiology and data analysis. B.M.S. and M.D.S. performed dendritic spine morphology  
3 experiments. A.J.H., C.M.B., K.B., Y.J.C., and A.T. performed molecular/biochemical experiments and  
4 data analysis. A.J.H. and C.M.B. performed statistical analyses.

5

6 **Disclosures: none**

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1 **Legends:**

2 **Table 1:** Summary of Clinical Features of MCHS Patients.

3 **Figure 1.** MCHS associated mutations in MEF2C disrupt DNA binding. (A) Schematic of the MEF2C  
4 protein with locations of MCHS mutations. MCHS mutations in green are further characterized (B-C).  
5 MCHS mutations that are newly described in this manuscript are denoted with “ \* ”. MCHS mutations not  
6 previously reported (personal communications) are denoted by “ \*\* “. The alternatively spliced beta  
7 (green) and gamma (blue) domains are shown. All MEF2C transcripts contain a C-terminal Nuclear  
8 Localization Sequence (NLS) that is disrupted by the frame-shift (fs) mutations. (B) Western blot of  
9 MEF2C wild-type (WT) and MCHS mutations in 293-T cells show that all MCHS mutations lead to protein  
10 expression. Arrows denote WT and mutant protein MEF2C bands. (C) Electrophoretic mobility shift assay  
11 (EMSA) using fluorescently labeled MEF2 response element (MRE) probe and MEF2C protein lysates  
12 from 293-T cells containing MEF2C mutations. MEF2C bound probe is shifted in the gel (denoted by “+”).  
13 Unbound fluorescent probe is denoted with a “-“. Only MEF2C WT binds to the fluorescently labeled  
14 MRE, while MCHS mutant proteins fail to bind the MRE probe (C). Quantification of bound probe is  
15 included (C); n=3. (D) Western blot of MEF2C from cortical lysates of control and *Mef2c*-Het mice. The  
16 black arrow denotes MEF2C WT and red arrows denote MEF2C DelEx2 (D,E). (E) Western blot of  
17 MEF2C WT and MEF2C DelEx2 from 293-T cells. (F) MEF2C DelEx2 fails to bind the MRE probe and  
18 does not interfere with MEF2C WT binding to MRE probes. “+” is bound probe. “-“ is unbound probe.  
19 Data are reported as mean  $\pm$  SEM. Also see Figure S1.

20 **Figure 2.** *Mef2c*-Het mice display multiple MCHS-relevant behaviors. (A) Three chamber social  
21 interaction test. Control mice spent significantly more time interacting with a novel animal over a novel  
22 object while the *Mef2c*-Het mice showed no preference for the novel object or the novel animal. (B)  
23 *Mef2c*-Het pups emitted fewer ultrasonic vocalizations (USVs) during maternal separation in early post-  
24 natal development. (C) Adult male *Mef2c*-Het mice produced fewer USVs than control mice in the  
25 presence of a female mouse in estrous. (D) Both control and *Mef2c*-Het mice have similar latencies to  
26 fall on an accelerating rotarod. (E,F) Male *Mef2c*-Het mice are hyperactive (E) and show increased jump  
27 counts (F). (G) *Mef2c*-Het mice spend significantly more time on the open arms of the elevated-plus  
28 maze. (H) *Mef2c*-Het mice have reduced response to shock. (I) Both control and *Mef2c*-Het mice increase

1 the number of active port entries (solid line) during sucrose self-administration. Dashed line represents  
2 inactive port entries. (J) Both control and *Mef2c*-Hets show similar active port entries during cue-induced  
3 reinstatement of sucrose seeking. Data are reported as mean  $\pm$  SEM. Statistical significance was  
4 determined by 2-way ANOVA (A,B,H,I) or unpaired t-test (C-G,J). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.005$ , n.s. =  
5 not significant. Number of animals (n) are reported in each graph for respective experiment. Also see  
6 Figure S2.

7 **Figure 3.** *Mef2c*-Het mice have alterations in cortical synaptic transmission. (A) Both control and *Mef2c*-  
8 Het mice have normal barrel fields in the somatosensory cortex, as reflected by VGlut2 staining. Scale  
9 bar=500  $\mu$ m. (B-F) Ex vivo recordings from organotypic slices were collected from pyramidal neurons  
10 within the barrel cortex field. (B) No changes were observed in mIPSC amplitude or frequency in the  
11 *Mef2c*-Het layer 2/3 pyramidal neurons. (C) Reduced EPSC amplitude and increased paired pulse  
12 facilitation (PPF) were observed in layer 2/3 *Mef2c*-Het neurons after stimulating input neurons from  
13 neighboring layer 2/3 neurons in adjacent barrel fields (horizontal inputs). (D) No changes in evoked  
14 EPSC amplitude or PPF were observed in layer 2/3 pyramidal neurons after stimulating input neurons  
15 from layer 4 (vertical inputs). "R" is recording electrode. "S" is stimulating electrode. (E,F) *Mef2c*-Het  
16 cortical pyramidal neurons have reduced mEPSC amplitude in layer 2/3 (E) and layer 5 (F), and increased  
17 mEPSC frequency in layer 2/3 (E). Data are reported as mean  $\pm$  SEM. Statistical significance was  
18 determined by unpaired t-test. \* $p < 0.05$ . Number of cells and animals, respectively, are reported in each  
19 graph. Also see Figure S3.

20 **Figure 4.** Differentially expressed genes in *Mef2c*-Het cortex. (A) Heatmap showing differentially  
21 expressed genes (DEGs) in *Mef2c*-Het cortex (p35-p40) compared with controls. In red, are genes with  
22 higher expression; in blue, are genes with lower expression. (B) *Mef2c*-Het DEGs are significantly  
23 enriched in genes associated with FMRP, ASD, or scored ASD (ASD\_1-3; high-confidence ASD genes)  
24 (see Methods). (C) *Mef2c*-DEGs are enriched in gene modules dysregulated in neuropsychiatric  
25 disorders, specifically the M1 and M6 modules. (D) qPCR validation of select *Mef2c*-Het DEGs  
26 associated with autism, microglia, or other cellular functions. Data are reported as mean  $\pm$  SEM (D).  
27 Statistical significance was determined by unpaired t-test (D). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.005$ ,



1 \*\*\*\* $p < 0.0005$ . See Methods for statistical analysis of A-C. Number of animals (n) is 4/genotype for RNA-  
2 Seq and 5/genotype for qPCR validation. Also see Figure S4.

3 **Figure 5.** *Mef2c*-Het mice exhibit increased Iba1 expression levels. (A,B) Representative images of Iba1-  
4 positive microglia in the SSctx in control (A) and *Mef2c*-Het mice (B). (C,E) *Mef2c*-Het mice have a right-  
5 shifted cumulative frequency distribution of mean Iba1 intensities in Iba1-positive cells (microglia) in the  
6 SSctx (C) and hippocampus (E) compared to controls. Gray line represents distribution of control cells  
7 and black line represents distribution of *Mef2c*-Het cells. (D,F) There is no difference in the cell soma  
8 volume of Iba1 positive cells (microglia) in the SSctx (D) or hippocampus (F) between controls and  
9 *Mef2c*-Het mice. (G) Fold changes of genes associated with microglial activation in controls and *Mef2c*-  
10 Hets. (H) *Mef2c*-Hets have an upregulation of genes expressed in postnatal immature, homeostatic, and  
11 embryonic microglia. Unless specified, data are reported as mean  $\pm$  SEM. Statistical significance  
12 determined by Kolmogorov–Smirnov test (C,E) or unpaired two-tailed nested t-test (D,F), unpaired two-  
13 tailed t-test (G) . \*\*\*\* $p < 0.0001$ . Sample sizes for each genotype are denoted on bars of or above each  
14 graph unless otherwise specified. Images (A,B) have contrast and brightness enhanced for ease of  
15 viewing. Images are modified equally for both genotypes. Also see Figure S5.

16 **Figure 6.** Cell type-selective behavior phenotypes in *Mef2c* conditional heterozygous (*Mef2c*-cHet) mice.  
17 (A-D) Behaviors in *Mef2c* cHet<sup>*Emx1*</sup> mice. (A) *Mef2c* cHet<sup>*Emx1*</sup> mice spend more time on the open arms of  
18 the elevated plus maze. (B,C) Male *Mef2c*-cHet<sup>*Emx1*</sup> mice are hyperactive (B) and show increased jump  
19 counts (C). (D) *Mef2c* cHet<sup>*Emx1*</sup> mice have normal social interaction. (E-H) Behaviors in *Mef2c* cHet<sup>*PV*</sup>  
20 mice. (E) *Mef2c* cHet<sup>*Pcp2*</sup> mice spend similar time on the open arms of the EPM. (F,G) Male *Mef2c* cHet<sup>*PV*</sup>  
21 mice are hyperactive (F) but show normal jump counts (G). (H) *Mef2c* cHet<sup>*PV*</sup> mice have normal social  
22 interaction. (I-L) Behaviors in *Mef2c* cHet<sup>*Cx3cr1*</sup> mice. (I) *Mef2c* cHet<sup>*Cx3cr1*</sup> mice are similar to controls in  
23 elevated plus maze. (J,K) Male *Mef2c* cHet<sup>*Cx3cr1*</sup> mice have normal activity (J) but show increased jump  
24 counts (K) compared to control mice. (L) *Mef2c* cHet<sup>*Cx3cr1*</sup> mice have a lack of preference for interacting  
25 with a novel mouse (social) over the novel object. Data are reported as mean  $\pm$  SEM. Statistical  
26 significance was determined by unpaired t-test (A-C,E-G,I-K) or 2-way ANOVA (D,H,L). n.s.=not  
27 significant, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.005$ , n.s. = not significant. Number of animals are reported in each  
28 graph. Also see Figure S6.

1 **Figures/Tables:**

2 **Table 1.** Summary of Clinical Features in MCHS Patients.

**Table 1. Overview of Clinical Characteristics associated with *MEF2C* variants**

	Newly described			Previously described by Zweier et al. [2010]	
<b>Demographics</b>					
Sex	F	F	F	F	F
Age at evaluation	13y11m	17m	4y3m	10y5m	3y
<b>Molecular findings</b>					
<i>MEF2C</i> variant	c.120_125dup p.D40_C41dup	c.90G>T p.K30N	c.137T>C p.I46T	c.80G>C p.G27A	c.113T>A p.L38Q
Inheritance of variant	Paternal (father mosaic)	<i>de novo</i>	Non-maternal	<i>de novo</i>	<i>de novo</i>
<b>Clinical findings</b>					
Height (percentile)	23rd	10-25th	25th	25-50th	NR
Weight (percentile)	25th	25-50th	40th	50th	25th
OFC (percentile)	NR	10-25th	25th	50th	9th
<b>Seizures</b>					
Seizures	Yes	Yes	Yes	Yes	Yes
Age of onset	8y	6m	21m	6m	10m
Global developmental delay	Yes	Yes	Yes	Yes	Yes
Speech	Few words	Absent	Few words	Absent	Absent
Tremors/tremulous	Yes	No	No	NR	NR
Hypotonia	Yes (resolved)	Yes	No	Yes	Yes
Brain MRI	Abnormal	Abnormal	Normal	Abnormal	Abnormal
Abnormalities noted	Areas of heterotopia in left lateral region, subtle but progressive abnormalities of white matter within frontal and parietal regions	Asymmetric appearance of hippocampi, asymmetric enlargement of temporal horn of left lateral ventricle	-	Mild under-myelination of insular cortices bilaterally	Generalized lack of white matter bulk and delay in myelin maturation
Repetitive movements	Yes	No	Yes	NR	NR
Breathing abnormalities	Yes	Yes	NR	Yes	No
Jugular fossa pit on anterior neck	No	No	No	NR	NR
High pain tolerance	Yes	NR	Yes	NR	NR
Sleeping difficulties	Yes	No	No	NR	NR
Social abnormalities	No	Yes	NR	NR	NR
Anxiety	No	No	NR	NR	NR
Autism spectrum disorder	No	No	Yes	NR	No
<b>Dysmorphic Findings</b>					
Midface	Hypoplastic	Full cheeks	Normal	NR	NR
Palpebral fissures	Upslanted	Upslanted with epicanthal folds	Normal	Downslanted	Upslanted
Nose	Sharp nasal tip, hypoplastic alae nasi, prominent columella	Normal	Normal	NR	NR
Mouth	Small	Bowed upper lip, small mandible	Dental crowding	Dental crowding, full upper lip	Bowed upper lip, widely spaced teeth
Chest	Mild pectus excavatum	Normal	Normal	NR	NR
Back	Mild lordosis	Normal	Normal	NR	NR
Digits	Long, increased space between toes 1 and 2	Normal	Normal	NR	NR
Other	-	Thin hair	-	Thick hair, large ears, fleshy ear lobes	Large ears, prominent ear lobes

NR=Not Reported

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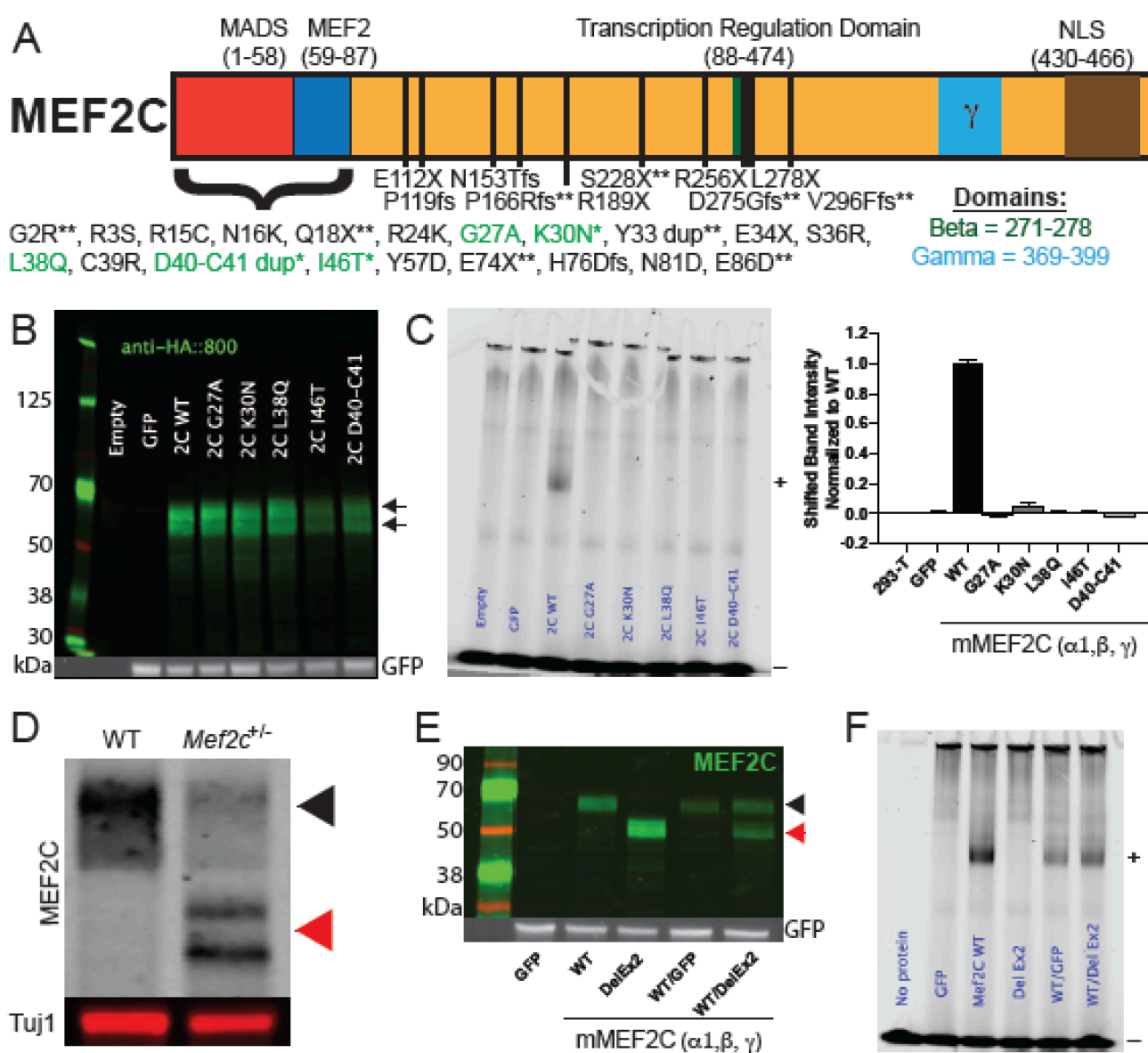
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1 **Figure 1.** MCHS-associated mutations in MEF2C disrupt DNA binding.



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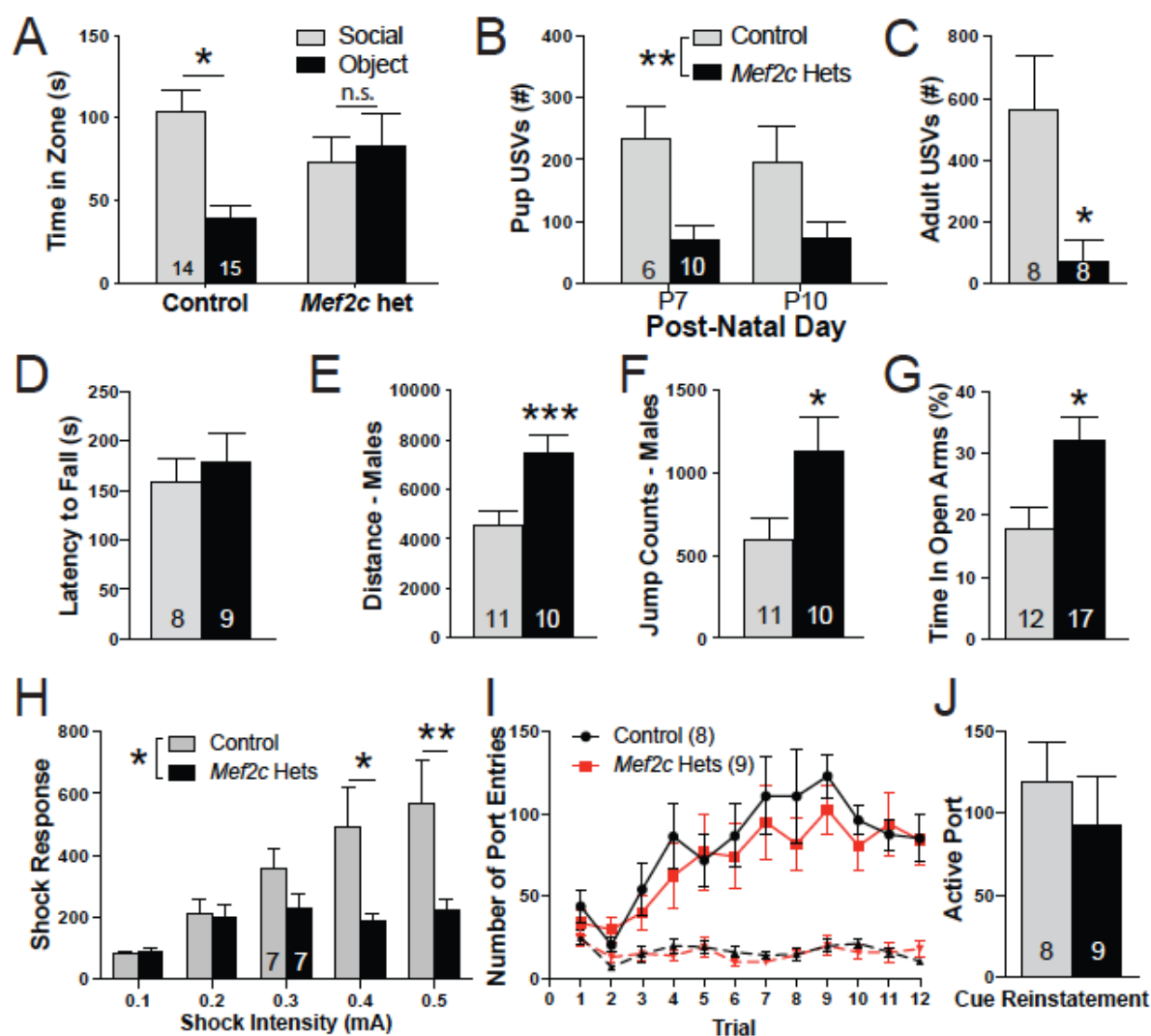
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1 **Figure 2. *Mef2c*-Het mice show MCHS-relevant behaviors.**



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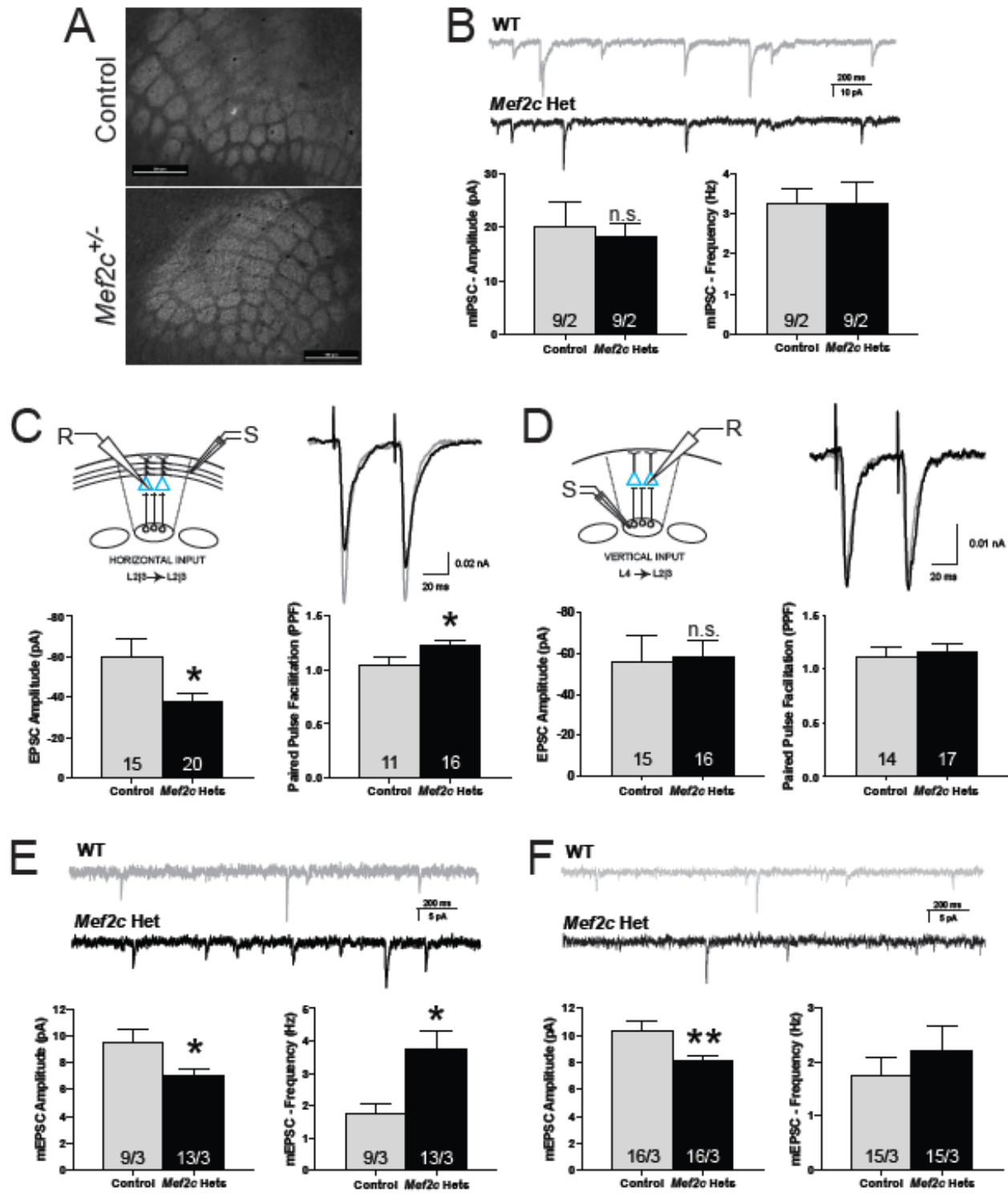
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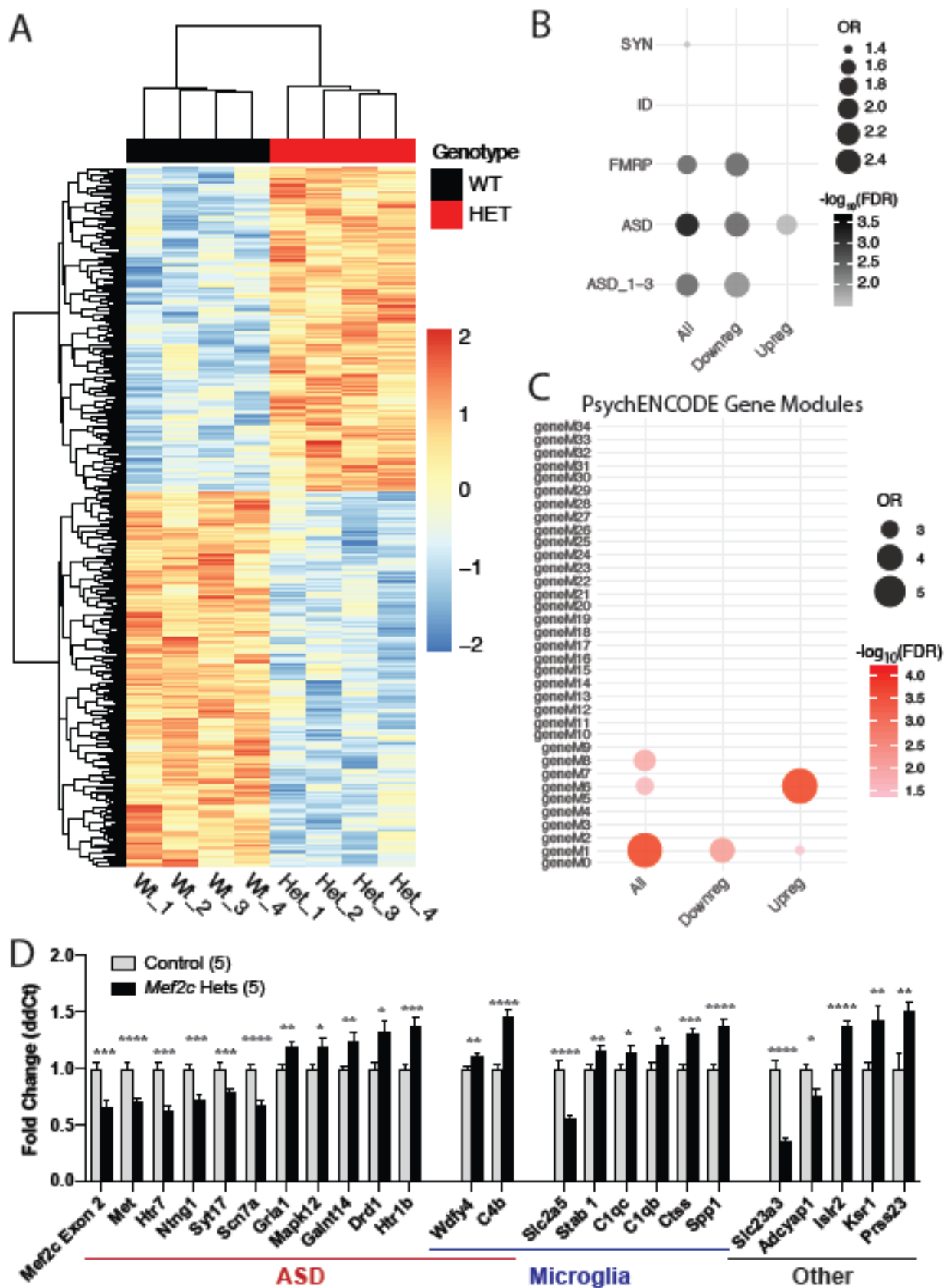
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1 **Figure 3. *Mef2c*-Het mice have changes in cortical synaptic transmission.**



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1 **Figure 4.** Differentially expressed genes in *Mef2c*-Het cortex

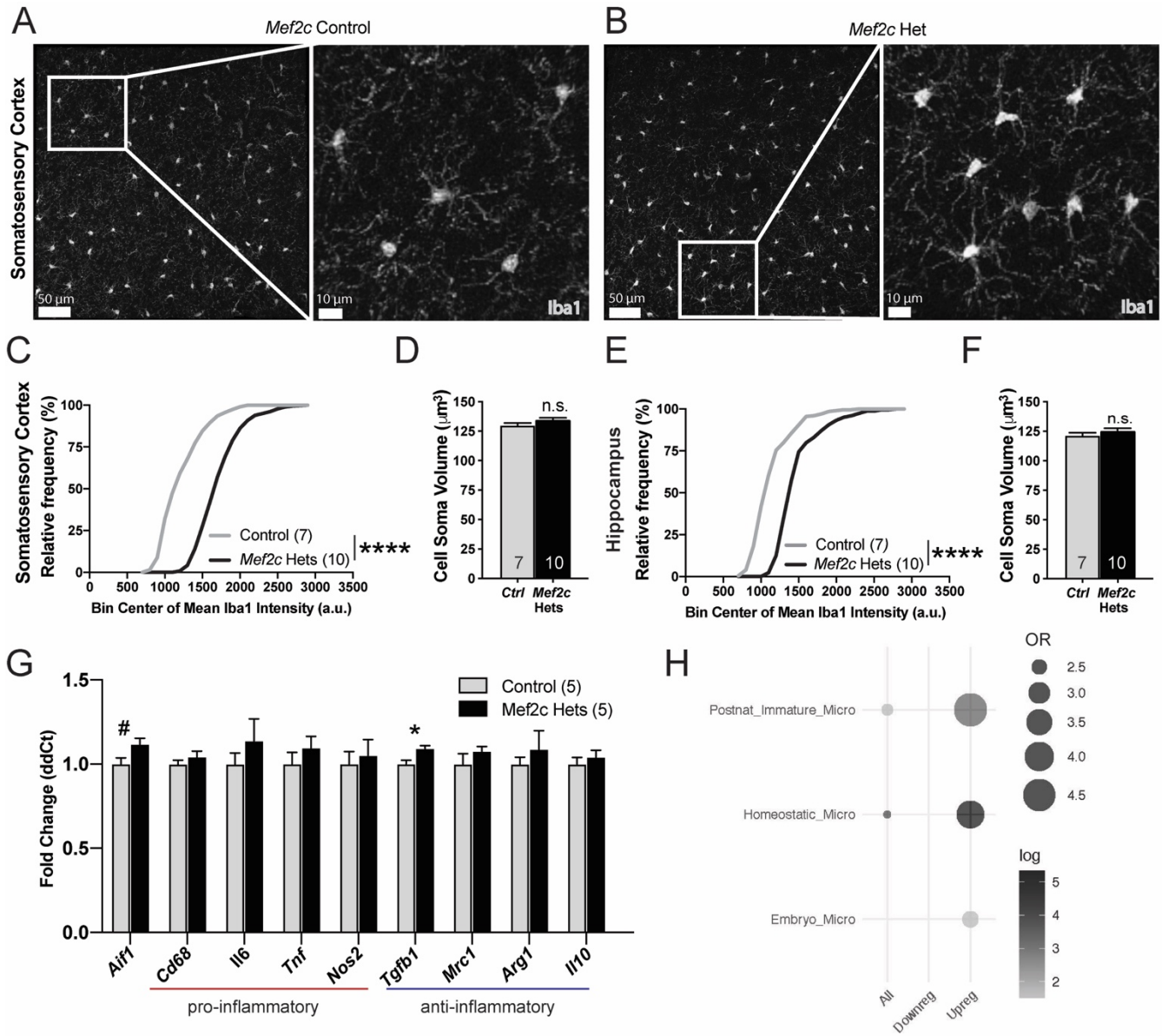


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1 **Figure 5. Microglia alterations in *Mef2c*-Het mice.**



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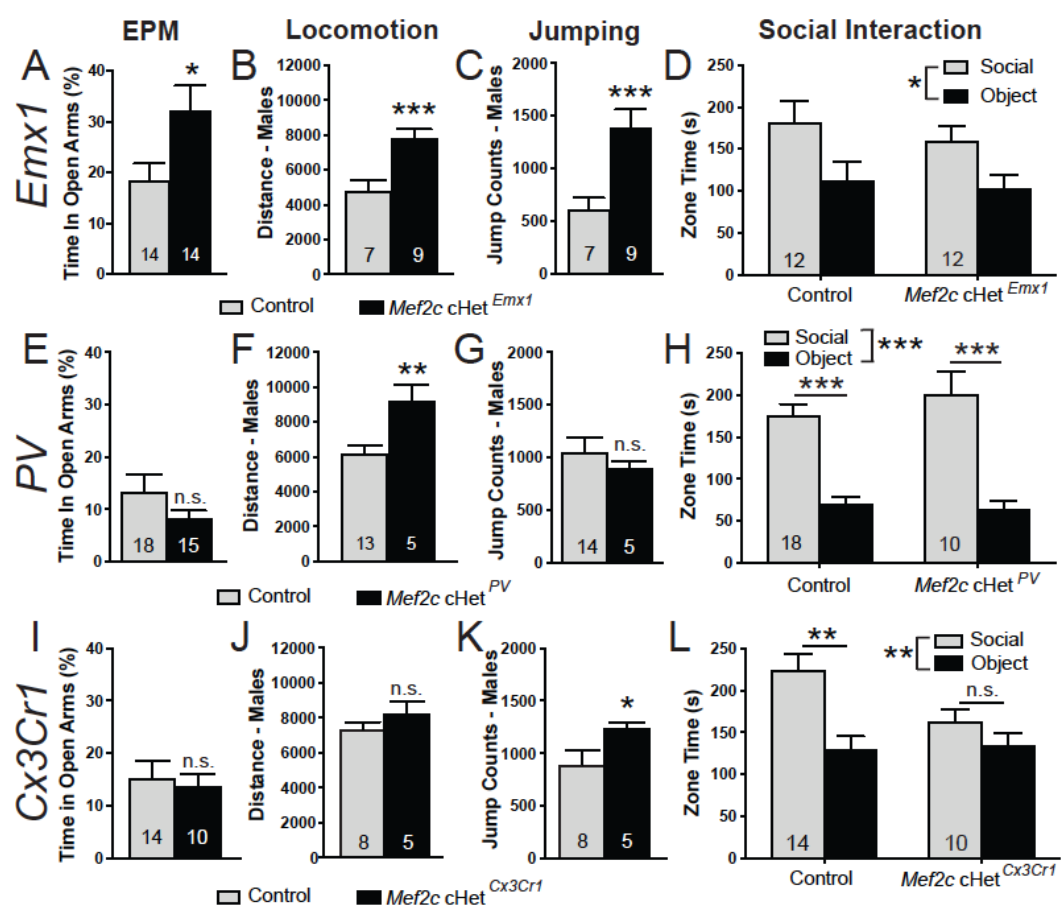
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1 **Figure 6.** Behaviors in neuron-specific *Mef2c*-cHet mice.



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1 **Supplemental Information:**

2 **Supplemental Figures:**

3 **Supplemental Table S1. RNA-Seq gene expression in control and *Mef2c*-Het cortex. See file**

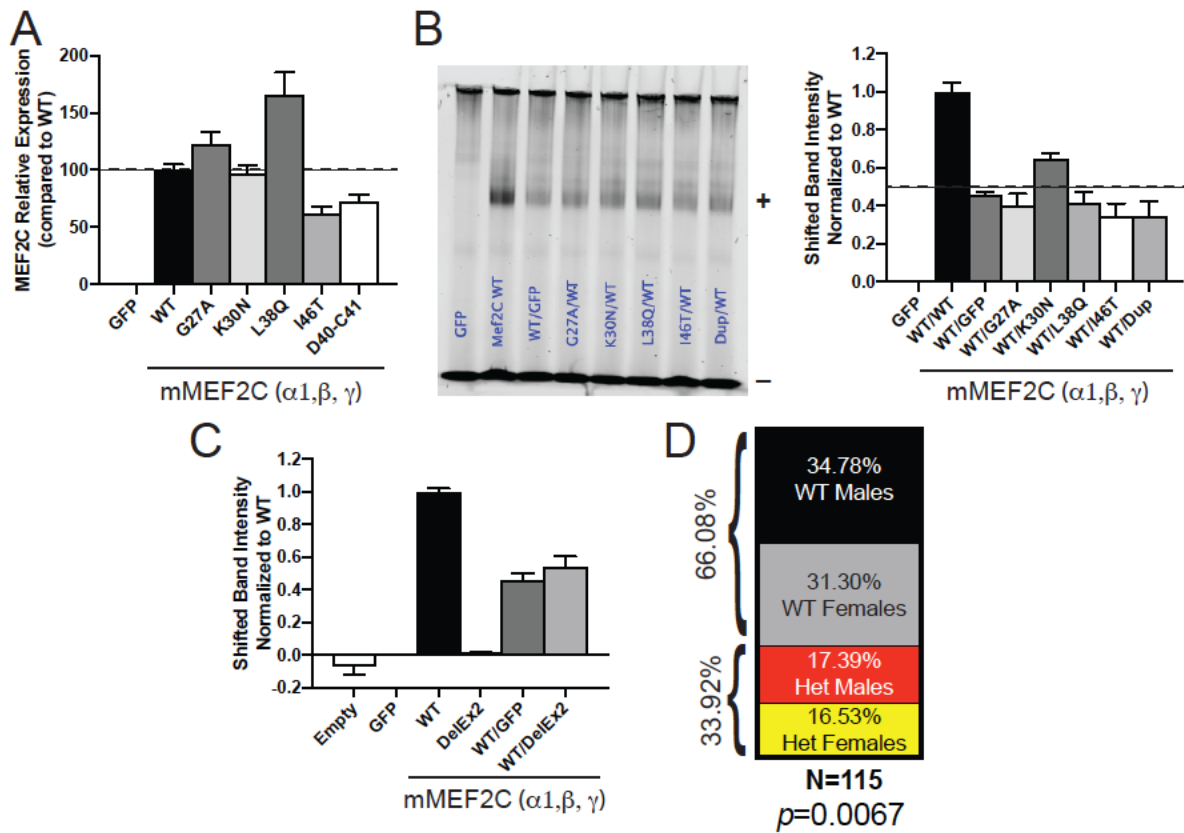
4 **“Mef2c het Database\_GenSet.”**

5 **Supplemental Table S2. *Mef2c* DEGs compared to single-cell RNA-Seq gene databases. See file**

6 **“Mef2c het Database\_single-cell.”**

7 **Supplemental Table S3. Primers for qPCR analysis of *Mef2c*-Het DEGs**

Gene:	Forward Primer:	Reverse Primer:
<i>Mef2c</i>	GTCGGCTCAGTCATTGGCTAC	CGCTACTCAGAGAGTACTCAG
<i>Mef2c Exon 2</i>	GTGCTGTGCGACTGTGAGAT	CTCCACAATGTCTGAGTTTGTC
<i>Met</i>	GTGAACATGAAGTATCAGCTCCC	TGTAGTTTGTGGCTCCGAGAT
<i>Htr7</i>	TGGGCTATGCAAACCTCTCTC	CAGCACAAACTCGGATCTCT
<i>Ntng1</i>	TGCTAAACACAGTCATTTGCGT	TGTTGTTCCAGTCTTACACTCAC
<i>Syt17</i>	GTCAGAGGTGCTATGAGTCCA	GGGGTCAAAGGAACATCGCT
<i>Scn7a</i>	TGTCTCCTCTAAACTCCCTCAG	TGCGTAAATCCCAAGCAAAGT
<i>Gria1</i>	CAAGTTTTCCCGTTGACACATC	CGGCTGTATCCAAGACTCTCTG
<i>Mapk12</i>	AGCCCTCAGGCTGTGAATCT	CATATTTCTGGGCCTTGGGT
<i>Galnt14</i>	GAATGTCTACCCAGAACTCAGGG	CCTTGGCACAGGGACTTAGC
<i>Drd1</i>	TGGCACAAGGCAAAACCTACA	CTGCTCAACCTCGTGTCAACA
<i>Htr1b</i>	CGCCGACGGCTACATTTAC	TAGCTTCCGGGTCCGATACA
<i>Wdfy4</i>	AAGTCAGTGTATGTGCTCACG	CTGCCCTTGAACATCGCTCT
<i>C4b</i>	GACAAGGCACCTTCAGAACC	CAGCAGCTTAGTCAGGGTTACA
<i>Slc2a5</i>	TCTCTTCCAACGTGGTCCCTA	GAGACTCCGAAGGCCAAACAG
<i>Stab1</i>	GGCAGACGGTACGGTCTAAAC	AGCGGCAGTCCAGAAGTATCT
<i>C1qc</i>	ACACATCGCATAACGGCCAA	AACATGTGGTTCGCAGAAGCTG
<i>C1qb</i>	TCACCAACGCGAACGAGAA	AAGTAGTAGAGGCCAGGCACCTT
<i>Ctss</i>	ATAAGATGGCTGTTTTGGATG	TTCTTTTCCAGATGAGACGC
<i>Spp1</i>	AGCAAGAACTCTTCCAAGCAA	GTGAGATTCGTCAGATTCATCCG
<i>Slc23a3</i>	TCTTCAACTTCAACTCACAT	ACAAAGGCAGAGATGAAC
<i>Adcyap1</i>	ACCATGTGTAGCGGAGCAAG	CTGGTCGTAAGCCTCGTCT
<i>Islr2</i>	GGCCACTGCGCCTACTCTATCT	GTCCCCCTGCTCCACATCTTCA
<i>Ksr1</i>	GCACCAAGTGCTCAGTGTCTA	CTGAAGCGTGGGTAGCTGTT
<i>Prss23</i>	GGTGAGTCCCTACACCGTTC	GGCGTCGAAGTCTGCCTTAG
<i>Aif1</i>	GTCCTTGAAGCGAATGCTGG	CATTCTCAAGATGGCAGATC
<i>Cd68</i>	CCACAGGCAGCACAGTGGACA	TCCACAGCAGAAGCTTTGGCCC
<i>Il6</i>	TACCACTTCACAAGTCGGAGGC	CTGCAAGTGCATCATCGTTGTTC
<i>Tnf</i>	GGTGCCTATGTCTCAGCCTCTT	GCCATAGAAGTATGAGAGGGAG
<i>Nos2</i>	GAGACAGGGAAGTCTGAAGCAC	CCAGCAGTAGTTGCTCCTCTTC
<i>Tgfb1</i>	TGATACGCCTGAGTGGCTGTCT	CACAAGAGCAGTGAGCGCTGA
<i>Mrc1</i>	GTTACCTGGAGTGATGGTTCTC	AGGACATGCCAGGGTCACCTTT
<i>Arg1</i>	CATTGGCTTGCAGACGTAGAC	GCTGAAGGTCTCTTCCATCACC
<i>Il10</i>	GGCAGAGAACCATGGCCAGAA	AATCGATGACAGCGCCTCAGCC



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2 **Figure S1.** (A) Quantification of MEF2C Western blot from 293-T cells containing MCHS mutations (Fig.  
 3 1B); n=3. (B) EMSA shows that MCHS-mutations in MEF2C do not disrupt WT MEF2C from binding to  
 4 the MEF2 response element (MRE); n=5. Quantification of MEF2C bound probe is reported (B). (C)  
 5 Quantification of MEF2C DelEx2 EMSA (Fig. 1F); n=4. (D) Genetic distribution of offspring from WT and  
 6 *Mef2c*-Het mice (n=115). Data are reported as mean  $\pm$  SEM. Statistical significance was determined by  
 7 Chi-squared test (D). Also see Figure 1.

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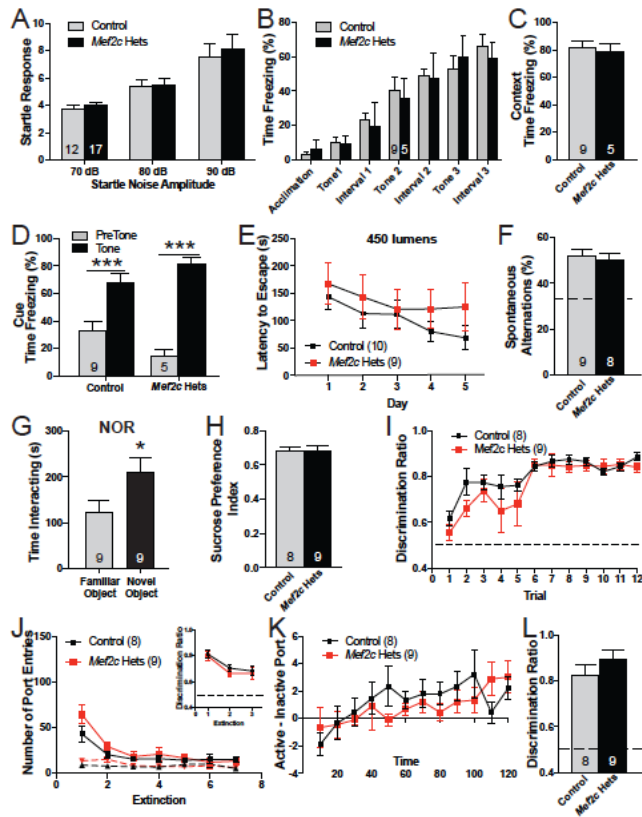
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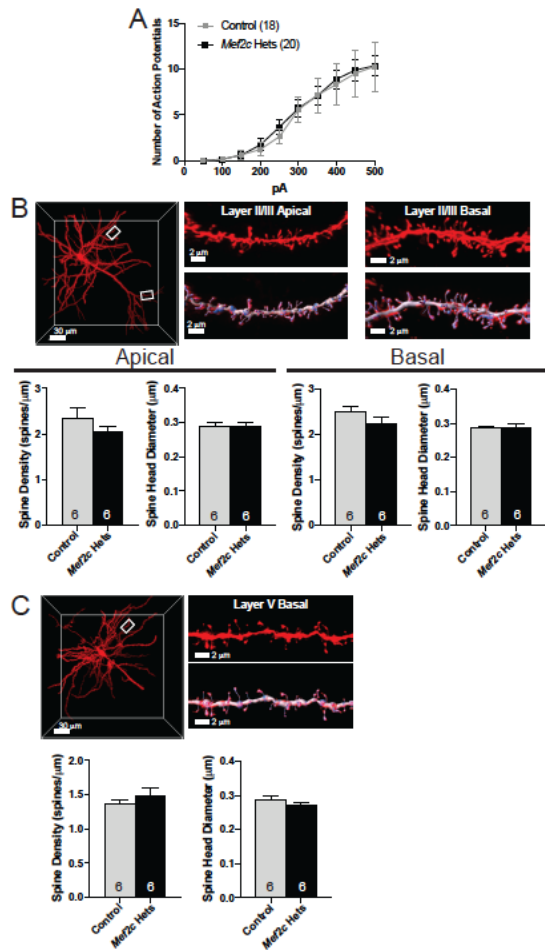




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2 **Figure S2.** *Mef2c*-Het mice have normal cognitive abilities. (A) There is no difference in acoustic startle  
3 response between *Mef2c*-Het and control mice. (B-D) Pavlovian Fear Conditioning. Both control and  
4 *Mef2c*-Het mice increase freezing with each tone/shock pairing during training (B) and show similar levels  
5 of freezing during the context (C) and cue (D) test. (E) No difference in latency to escape a very bright  
6 (450 lumens) Barnes Maze. (F) Both control and *Mef2c*-Het mice have similar spontaneous alternations  
7 in the Y-maze. (G) Novel object recognition. *Mef2c*-Het mice (n=9) interacted more with a novel object  
8 than a familiar object. (H) Both genotypes show a similar preference for 1% sucrose solution. (I-L)  
9 Sucrose Self-Administration Task. (I) Discrimination ratio during sucrose self-administration. (J) Number  
10 of active (solid line) and inactive (dashed line) port entries during extinction. Control and *Mef2c*-Het mice  
11 can recall the active port on the first day of extinction, and both genotypes show similar extinction rates  
12 (J) and discrimination ratio (insert). (K) Discrimination ratio during the first day of sucrose self-  
13 administration. (L) Both control and *Mef2c*-Het mice have high discrimination ratios during cue-induced  
14 reinstatement of sucrose seeking. Data are reported as mean  $\pm$  SEM. Statistical significance was  
15 determined by 2-way ANOVA (A,B,D,E,I-K) or unpaired t-test (C,F-H,L). \* $p < 0.05$ , \*\*\* $p < 0.005$ . Number  
16 of animals (n) are reported in each graph for respective experiment. Also see Figure 2.





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2 **Figure S3.** (A) Both control and *Mef2c*-Het cortical pyramidal neurons have similar numbers of action  
3 potentials evoked by increasing current injections (500ms, 50pA steps) recorded in current clamp. (B,C)  
4 Representative image of a Dil filled cortical pyramidal neuron (B=layer 2/3; C=layer 5) and representative  
5 apical and basal dendritic segments used for quantifying dendritic spines. Both control and *Mef2c*-Het  
6 mice have similar dendritic spine density and spine head diameter on apical and basal dendrites. Data  
7 are reported as mean  $\pm$  SEM. Statistical significance was determined by 2-way ANOVA (A) or unpaired  
8 t-test (B,C). Number of cells (A) or animals (B,C) are reported in each graph for respective experiment.  
9 Scale bar=30  $\mu\text{m}$  (neuron) or 2  $\mu\text{m}$  (dendritic stretch). Also see Figure 3.

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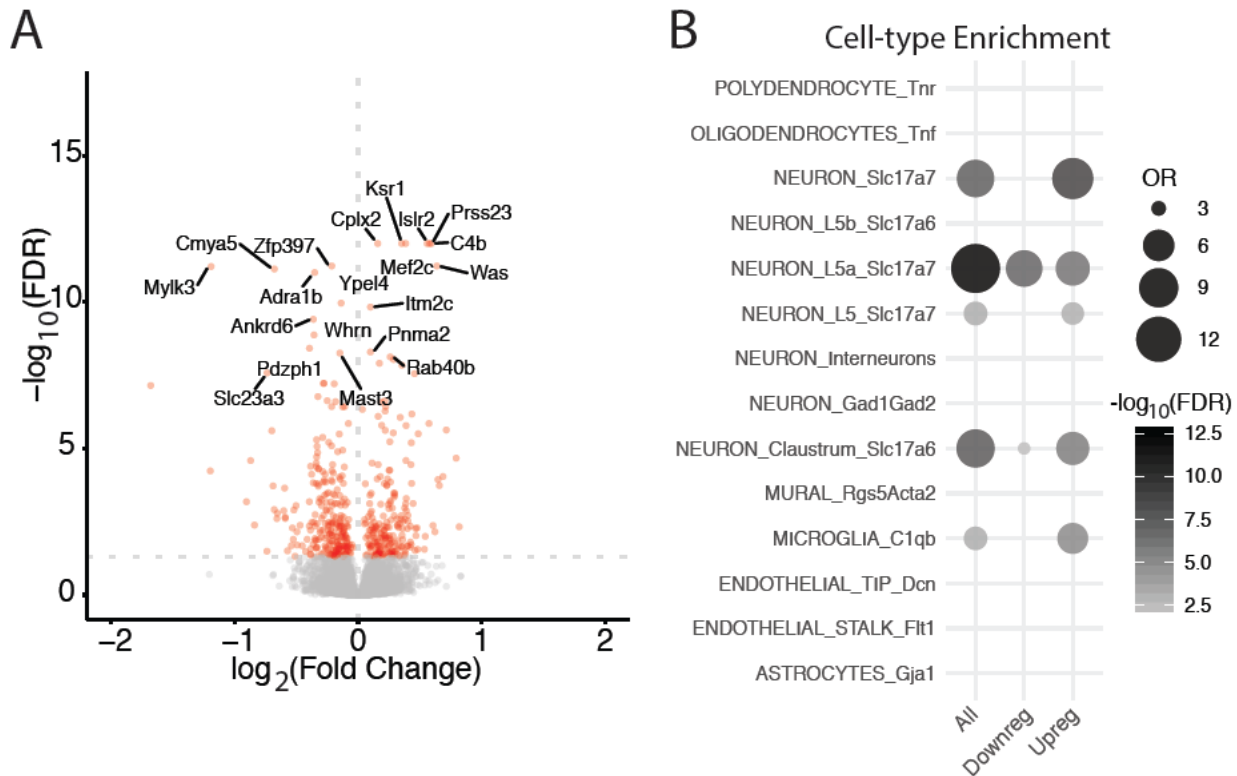
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2 **Figure S4.** Differentially expressed genes in *Mef2c*-Het cortex. (A) Volcano plot of *Mef2c*-Het DEGs  
3 shows genes are both up- and down-regulated in the cortex of *Mef2c*-Het mice. (B) Bubble-plot of *Mef2c*-  
4 Het DEGs are enriched for genes expressed in neurons and microglia. See Methods for statistical tests.  
5 Number of animals is 4/genotype. Also see Figure 4.

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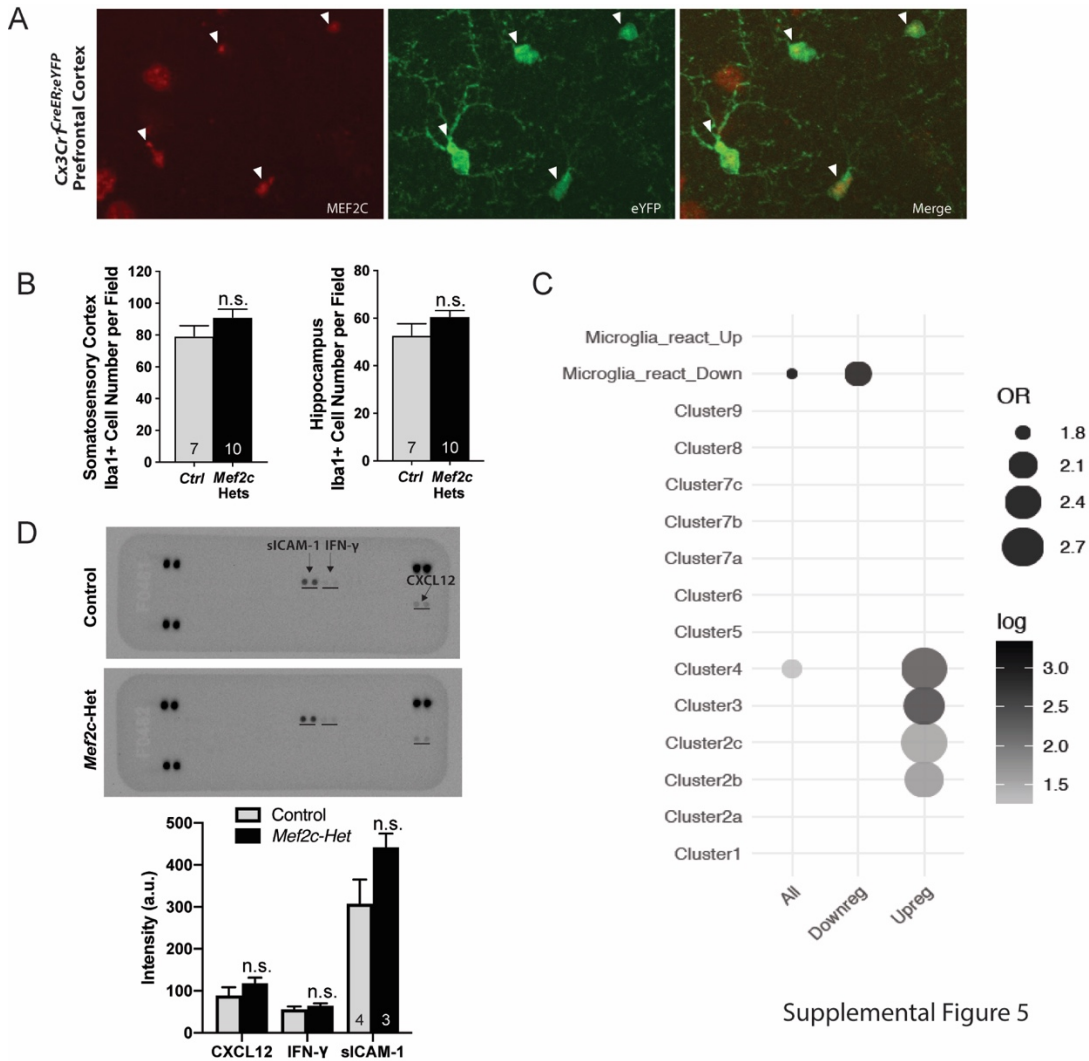
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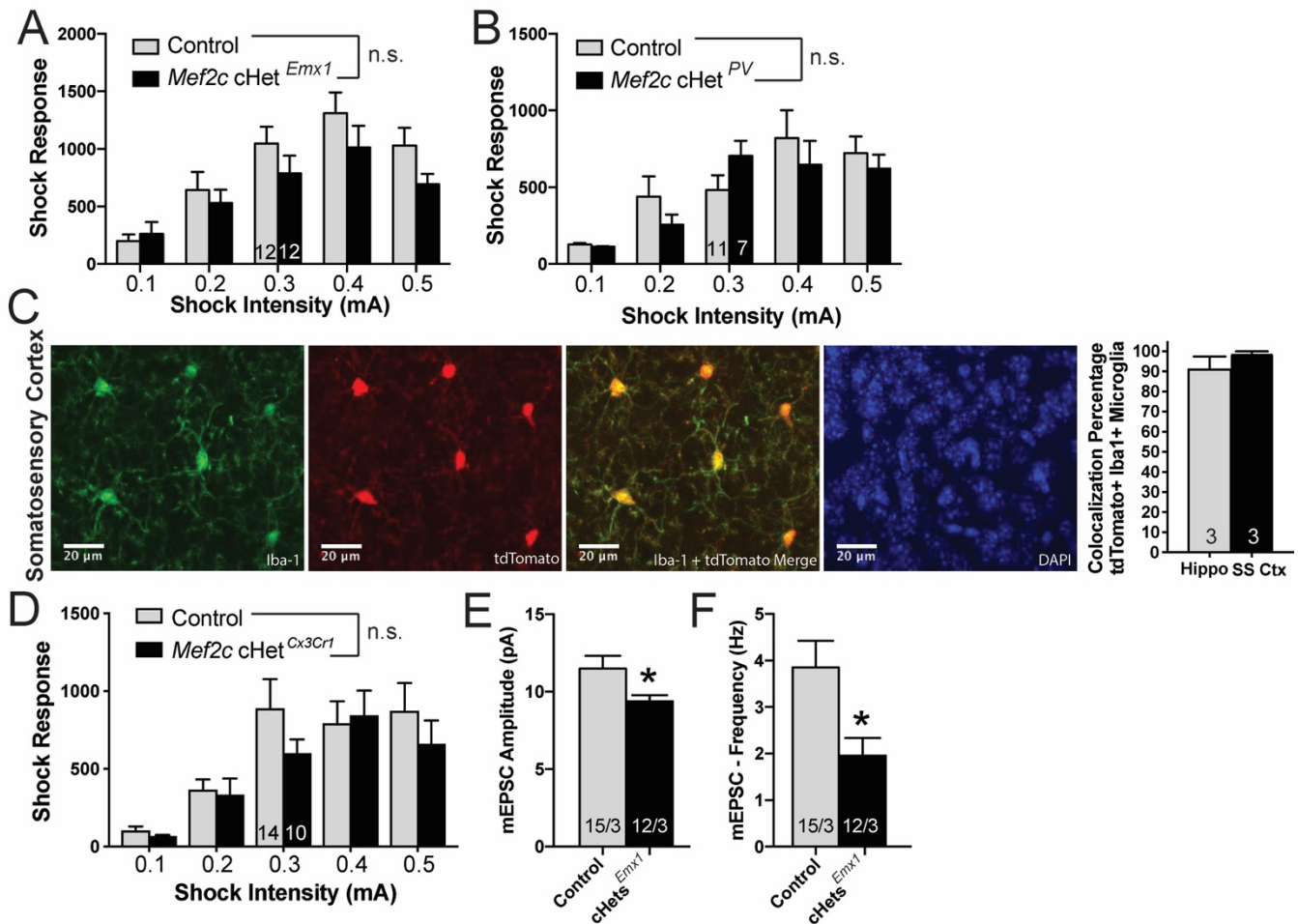
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Supplemental Figure 5

1  
2 **Figure S5.** (A) MEF2C is expressed in microglia. Using a microglia reporter mouse (*Cx3Cr1<sup>CreER</sup>;eYFP*),  
3 immunohistochemistry reveals that MEF2C (red) is expressed in microglia (green) under basal  
4 conditions. (B) Microglial cell density in the somatosensory cortex and dentate gyrus of the hippocampus,  
5 quantified via Iba1 staining of microglia, is not significantly different between *Mef2c*-Hets and controls.  
6 (C) Enrichment of differentially expressed genes in *Mef2c*-Hets are upregulated in cluster 2b (G-phase  
7 proliferative cluster), cluster 2c (M-phase proliferative cluster), and cluster 3 (metabolically active  
8 microglia at E14.5), and cluster 4 (microglia associated with unmyelinated axon tracts at (P4/5)). (D)  
9 Cytokine antibody array analysis for assessing neuroinflammation in *Mef2c*-Hets and controls at P35-  
10 P40. Data are reported as mean  $\pm$  SEM. Statistical significance was determined by unpaired t-test (B,D).  
11 Number of animals (B,D) are reported in each graph for respective experiment. Also see Figure 5.  
12



1

2 **Figure S6.** (A,B) *Mef2c* cHet<sup>Emx1</sup> (A) and *Mef2c* cHet<sup>PV</sup> (B) mice have normal response to shock. (C)  
 3 Recombination efficiency after p1-p3 tamoxifen treatment in *Cx3Cr1*<sup>CreER;eYFP</sup>; Ai14 mice. tdTomato  
 4 expression suggests recombination in these Iba1-positive microglia cells. (D) *Mef2c* cHet<sup>Cx3Cr1</sup> mice are  
 5 similar to controls in shock response. (E,F) *Ex vivo* recordings from organotypic slices were collected  
 6 from pyramidal neurons within the barrel cortex field of *Mef2c* cHet<sup>Emx1</sup> mice. Neurons from *Mef2c*  
 7 cHet<sup>Emx1</sup> show reduced mEPSC amplitude (D) and frequency (E) compared to controls. Data are reported  
 8 as mean ± SEM. Statistical significance was determined by 2-way ANOVA (A-B,D) or unpaired t-test  
 9 (E,F). Number of animals (A-B,D) or cells/animals (E,F), respectively, are reported in each graph for  
 10 respective experiment. Also see Figure 6.

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## 1 Supplemental Methods

### 2 Patients

3 Patients with developmental delay and a significant variant in the MEF2C gene were selected for this  
4 study. These patients were seen for clinical genetics evaluations and data from these visits were gathered  
5 from records review. Internal informed consent to publish data was obtained for each subject.

### 7 Animals

8 Mice (*Mus musculus*) were group housed (2-5 mice/cage; unless specified) with same-sex littermates  
9 with access to food and water ab libitum on a 12 hour reverse light-dark cycle. *Mef2c*<sup>+/-</sup> (*Mef2c*-Het) mice  
10 were initially generated by crossing *Mef2c*<sup>fl/fl</sup> (RRID:MGI:3719006)(Arnold et al., 2007) mice with *Prm1*-  
11 *Cre* (Jackson Laboratory #003328) to induce germline recombination of *Mef2c*. *Mef2c*<sup>+/-</sup>; *Prm1*-*Cre* were  
12 crossed with C57BL/6J to remove *Prm1*-*Cre*, and test mice were generated from *Mef2c*<sup>+/-</sup> mice crossed  
13 with C57BL/6J mice. *Mef2c* conditional het mice were generated by crossing *Mef2c*<sup>fl/fl</sup> mice with  
14 heterozygous cell-type specific Cre mice (*Emx1*-*Cre*(Iwasato et al., 2008); *Pcp2*-*Cre* (Jackson Laboratory  
15 #004146)(Barski et al., 2000); *PV*-*Cre* (Jackson Laboratory #017320) to generate *Mef2c*<sup>fl/+</sup>; *Cre*  
16 conditional heterozygous (*Mef2c* cHet) mice that were compared to their Cre-negative littermates.  
17 *Cx3Cr1*-*Cre* *Mef2c* conditional heterozygous (*Mef2c* cHet<sup>Cx3Cr1</sup>) mice were generated by crossing  
18 *Cx3Cr1*<sup>creER/creER</sup> males (Jackson Laboratory #021160)(Parkhurst et al., 2013) with *Mef2c*<sup>fl/+</sup> females to  
19 produce *Cx3Cr1*<sup>creER/+</sup>; *Mef2c*<sup>fl/+</sup> (experimental) and *Cx3Cr1*<sup>creER/+</sup>; *Mef2c*<sup>+/+</sup> (control) mice. Experimenters  
20 were blinded to the mouse genotype during data acquisition and analysis. Experiments were  
21 independently replicated, and the total number of animals/cells were reported in the representative  
22 figures. All procedures were conducted in accordance with the Institutional Animal Care and Use  
23 Committee (IACUC) and NIH guidelines.

25 **EMSA:** Electrophoretic mobility shift assay (EMSA) was performed as previously described  
26 (Pulipparacharuvil et al., 2008) with modifications. Briefly, proteins were isolated from 293-T cells 48  
27 hours after transfection of pA1T7α::*Mef2c* variants in a non-denaturing EMSA cell lysis buffer (20 mM  
28 tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 10 mM NaF, 0.5% Nonidet P-40, 1 μM

1 cyclosporin A, 1X Complete Protease Inhibitor cocktail (Roche)), and quantified using the DC protein  
2 assay kit (BioRad). Isolated proteins (10  $\mu$ g) were incubated with fluorescent-tagged (Infrared Dye 700)  
3 Mef2 Response Element (IR700::MRE) probes for 60 minutes at room temperature (RT) and resolved in  
4 a non-denaturing acrylamide gel (50 mM tris-HCl (pH 7.5), 380 mM glycine, 2 mM EDTA, 4%  
5 acrylamide/bis-acrylamide (BioRad)). Gels were imaged using the Li-Cor Odyssey CLx, and fluorescence  
6 was quantified by Li-Cor Image Studio v3.1.4. Data reported represent the mean from at least 3 biological  
7 replicates.

8

9 **Immunoblotting:** EMSA proteins were denatured by adding 4X sample buffer (+DTT, + $\beta$ ME) to samples  
10 in EMSA cell lysis buffer and boiling for 10 minutes. Somatosensory cortex was isolated from adult (8  
11 weeks old) male mice and frozen on dry ice. Tissues were sonicated on ice in an SDS lysis buffer (1%  
12 (w/v) SDS, 300 mM sucrose, 10 mM NaF (Sigma), 50 mM HEPES (Sigma), and 1X Complete Protease  
13 Inhibitor cocktail (Roche)), boiled for 10 minutes, then centrifuges at 16,000 x g for 10 minutes. Total  
14 protein concentration was determined by the DC protein assay kit (BioRad). For western blots, 20  $\mu$ g of  
15 total protein was resolved using 10% SDS-PAGE (BioRad). Proteins were transferred to Immobilon-FL  
16 PVDF (Millipore), blocked in Odyssey blocking buffer (Li-Cor) for 2 hours, and incubated overnight with  
17 either anti-HA (Sigma-Aldrich H6908; 1:2000) and anti-GFP (Aves Labs GFP-1020; 1:2000) for EMSA  
18 proteins, or anti-Mef2c (AbCam ab197070; 1:2500) and anti-Neuronal class III  $\beta$ -tubulin (Tuj1, Covance;  
19 1:10,000) antibodies for cortical proteins. Blots were developed with Odyssey CLx Western blot system  
20 (Li-Cor Biosciences) (HA, MEF2C, and TUJ1) or ChemiDoc MP Imaging System (BioRad) (GFP).

21

22 **Data Acquisition:** All experiments were independently replicated at least twice (typically 3-4 times). The  
23 numbers of animals/neurons/dendritic stretches are reported in each figure, and these numbers were  
24 estimated based on previous reports. Outliers were determined using GraphPad's Outlier calculator  
25 (Grubb's) and excluded from data analysis.

26

27 **Mouse Behavior Testing:** For all behavior tests, test mice were acclimated to transport and handling  
28 for at least 3 days prior to testing. Before each test, mice were acclimated after transport for >30 minutes



1 prior to testing. Behaviors in *Mef2c*-Het mice were compared to control littermates tested on the same  
2 day. All behavioral tests were conducted using young adult mice (8-12 weeks), except juvenile  
3 communication (USV) recordings. Both male and female animals were included, except for adult  
4 ultrasonic vocalizations recordings. All behavior tests were conducted during the dark-phase (active-  
5 phase), and the experimenters were blind to genotypes.

6  
7 **Behavior Data Analysis:** All data are presented as mean  $\pm$  SEM. All comparisons were between  
8 littermates using appropriate two-sided statistical tests (specified in figure legends). Normal distribution  
9 of the data was assumed. Outliers were determined using GraphPad's outlier calculator (Grubb's) and  
10 excluded from analysis. *P*-values were calculated with unpaired *t*-test (two-tailed) or two-way ANOVA  
11 followed with Sidak's multiple comparisons post-hoc test using GraphPad Prism, with specific tests  
12 described in figure legends.

13  
14 **Social Interaction:** Mice were acclimated to the 3-arena sociability apparatus (Stoelting #60450) for 10  
15 minutes. After acclimation, test mice were removed from the arena, and a novel, conspecific mouse and  
16 a novel object (medium black paper binder) were placed in a holding chamber within the side arenas.  
17 Test mice were returned to the arena and recorded for 10 minutes using ANY-maze behavior tracking  
18 software (Stoelting). Time is defined as time spent in each arena.

19  
20 **Ultrasonic vocalization recordings:** Social ultrasonic vocalizations (USVs) were recorded from adult  
21 mice as previously described (Ey et al., 2013; Harrington et al., 2016). Briefly, ovariectomized female mice  
22 (C57BL/6; Jackson Labs) were injected with 15- $\mu$ g estradiol 48 hours prior to testing and 1-mg  
23 progesterone 4 hours before testing to induce estrous. Test mice (8-12 week old male mice) were  
24 acclimated to a clean home-cage in a sound attenuated chamber for 5 minutes. After acclimation, an  
25 estrous female was introduced into the holding chamber with the male test mouse, and USVs were  
26 recorded for 5 minutes using Avisoft UltraSoundGate equipment (UltraSoundGate 116Hb with Condenser  
27 Microphone CM16; Avisoft Bioacoustics, Germany). USVs were analyzed using Avisoft SASLab Pro  
28 (Avisoft Bioacoustics) using a 20 kHz cutoff. Distress USVs were recorded from juvenile mice (pups) as

1 previously described(Ey et al., 2013; Harrington et al., 2016; Scattoni et al., 2008). Briefly, individual pups  
2 of both sexes were identified with long-lasting subcutaneous tattoos (green tattoo paste; Ketchum) on  
3 the paws on post-natal day 4 (P4). Pups of both sexes were recorded in a random order in a small,  
4 sound-attenuated chamber following separation from dam and littermates. USVs were recorded for 3  
5 minutes on post-natal days 7 and 10. USVs were quantified using Avisoft SASLab Pro (Avisoft  
6 Bioacoustics, German) with experiments blind to genotype.

7

8 **Rotarod test:** Mice were placed on the roller in a Rotarod apparatus (Ugo Basile Apparatus #47600) for  
9 a 2-minute training session with a rotation of 4 rpm or 8 rpm and replaced if mouse falls during training  
10 session. On the next day, test mice were returned to the roller, and the speed steadily increases from 4-  
11 40 rpms over 5 minutes. The latency and rpm for the mouse to fall off the roller were recorded. Each  
12 animal receives four testing sessions.

13

14 **Locomotor Activity:** Test mice were placed inside the Open Field Activity, Infrared Photobeam Activity  
15 Test Chamber (Med Associates), where an array of photobeams measures the mouse's locomotor  
16 activity and jumping. Activity was monitored in the dark for one hour, and data are presented as the total  
17 activity during the hour.

18

19 **Elevated Plus Maze (EPM):** Mice were introduced in the center of the elevated plus maze (Stoelting  
20 #60140) in white light (100 lux) and recorded for 5 minutes using ANY-maze behavior tracking software  
21 (Stoelting) with center-point detection. Data are reported as the percent of time spent in the open areas.

22

23 **Fear Conditioning Test:** Fear conditioning was performed as previously described(Wehner and  
24 Radcliffe, 2004). Briefly, test mice were placed in a fear conditioning chamber (Med Associates) and  
25 allowed to explore the arena for 2 minutes, after which a loud auditory stimulus (30 secs; 90 dB) that co-  
26 terminates with a 2-second mild foot-shock (0.5 mA) was presented to the animal. The mice were  
27 exposed to 3 tone/shock pairing with a 1-minute interval separating each tones/shock. The next day,  
28 animals were returned to the chamber and behavior (freezing) in the context, in a new context, and with



1 the audible tone played in a new context is recorded with a video-tracking system (Video Freeze V2.7;  
2 Med Associates). Data are presented as percent of time the mouse is immobile.

3

4 **Barnes Maze:** Barnes maze was conducted as previously described (Rosenfeld and Ferguson, 2014)  
5 with modifications. Briefly, on the initial trial, mice were acclimated to the escape chamber for 2 minutes  
6 before testing. During testing, mice were introduced to the center of the Barnes Maze (Stoelting #60170)  
7 in bright white light (250 or 450 lux) with 4 distinct spatial cues evenly distributed around the arena. Mice  
8 were allowed to freely explore the arena for 5 minutes or until the mice found and entered the escape  
9 chamber. Latency to escape was recorded for each trial. Mice failing to find the escape chamber at the  
10 end of the trial were guided to the escape hole. All mice were left in the escape chamber for 2 minutes  
11 before being returned to their home cage. Each mouse was tested twice a day, and data reflect the  
12 average latency to enter the escape hole of the 2 trials per day.

13

14 **Y-maze:** Mice were introduced into one arm of a Y-maze (Stoelting #60180) with minimal white light (30  
15 lux), and the mice were video tracked using ANY-maze behavior tracking software (Stoelting) for 5  
16 minutes. Correct alternations were considered when the mouse entered a series of 3 different arms  
17 without re-entering a previously explored arm.

18

19 **Novel Object Recognition (NOR):** Mice were acclimated for 10 minutes to an open field arena (OF;  
20 44cm<sup>2</sup>) in dim light (30 lux) the day before testing. On test day, mice were acclimated to the OF for 5  
21 minutes before objects were introduced. Mice were presented with 2 identical objects located on opposite  
22 sides of the OF arena and allowed to explore the objects for 10 minutes. One initial object was replaced  
23 with a novel object, and the mice were allowed to explore the objects for 10 minutes. Mice were recorded  
24 and analyzed using ANY-maze behavior tracking software (Stoelting). Interactions were considered when  
25 the center of the mouse was within 8 cms from the center of the object.

26

27 **Sucrose preference:** Test mice were singly housed and provided 2 identical ball-bearing sipper-style  
28 bottles to drink. Mice were acclimated to the 2 bottles for 4 days, where both bottles contained water on

1 days 1 and 3 or sucrose solution on days 2 and 4. On days 5-8, mice were presented with 2 bottles, one  
2 with water and one with sucrose (1% (w/v)). Daily, the consumption of water and sucrose/quinine was  
3 measured, and the bottle position was altered to avoid potential side bias(Renthal et al., 2007). Data is  
4 presented as (solution consumption – water consumption) / total consumption = Preference Index.

5  
6 **Sucrose Self-Administration (SSA):** Sucrose self-administration (SA) was performed as previously  
7 described(Taniguchi et al., 2017). Briefly, mice were introduced to an operant conditioning chamber (Med  
8 Associates) at the same time each day during the dark cycle (active-phase). Both a light above the active  
9 nose poke hole and the house light indicated that sucrose was available. After an active hole nose poke,  
10 the availability lights went off and an internal light in the nose poke hole was activated. Active nose pokes  
11 immediately delivered a sucrose pellet (15 mg; TestDiet) and was followed by a 10 s time-out period.  
12 Inactive hole nose pokes did not have any consequences. After 12 days of acquiring, the mice entered a  
13 7-day abstinence phase in their home cages and were not exposed to the operant conditioning chamber.  
14 Following abstinence, the mice were placed back in the operant chamber for 2 hours, where the sucrose  
15 pellets and cues were not present. Following 7 days of extinction, mice were re-introduced to the operant  
16 chamber and the availability cues and reward delivery cue (but no sucrose reward) were presented (cue-  
17 induced reinstatement). The number of active and inactive hole nose pokes were recorded during each  
18 session. Discrimination ratio is reported as number of active port entries / total number of port entries  
19 (active + inactive).

20  
21 **Shock and Acoustic Startle Response:** Shock and acoustic startle response was performed as  
22 previously described(Harrington et al., 2016). Briefly, shock and startle responses were measured using  
23 Startle Reflex System and Advanced Startle software program (Med Associates). Mice were placed in  
24 Plexiglas and wire grid animal holders (ENV-264C) attached to a load cell platform (PHM-250) contained  
25 within a sound-attenuated chamber. For shock sensitivity, foot shocks (0.1 – 0.5 mA) were delivered by  
26 S/A Aversive Stimulators (ENV-414S) connected to the wire grid floors of the animal holder. For acoustic  
27 startle, mice were exposed to 5 white-noise pulses/amplitude (38ms) at 3 different amplitudes (70, 80,  
28 90 dB) in a randomized order with variable inter-trial intervals (10-20s). Displacements of the load cell

1 stabilimeter were converted into arbitrary units by an analog-to-digital converter interfaced to a personal  
2 computer.

3

4 **RNA Isolation and Reverse Transcription PCR.** Cortical tissue from p35-p40 mice (2 males and 2  
5 females per genotype) were rapidly dissected and frozen at -80°C. Samples were thawed in TRIzol  
6 (Invitrogen), homogenized, and processed using the miRNeasy Mini Kit (Qiagen) according to  
7 manufacturer's protocol. Total RNA was reverse-transcribed using Superscript III (Invitrogen) with  
8 random hexamers following manufacturer's protocol. Quantitative real-time PCR was performed by the  
9 CFX96 qPCR instrument (Bio-Rad) using iTaq Universal SYBR Green Supermix (Bio-Rad) and primers  
10 specific to each target gene (Table S3). GAPDH was used to normalize gene expression in each sample.

11

12 **RNA Sequencing.** Total RNA was isolated from whole cortex of p35-p40 mice as described above.  
13 Sequencing was performed by BGI Americas Corporation (Cambridge, MA) using polyA mRNA isolation,  
14 directional RNA-seq library preparation, and the BGISEQ-500 platform with 150bp paired-end reads  
15 using DNA Nanoball (DNB) technology.

16

17 **RNA-seq mapping, QC and expression quantification.** Reads were aligned to the mouse mm10  
18 reference genome using STAR 2.7.2a (Dobin et al., 2013) with the following parameters: "--  
19 *outFilterMultimapNmax 10 --alignSJoverhangMin 10 --alignSJDBoverhangMin 1 --*  
20 *outFilterMismatchNmax 3 --twopassMode Basic*". For each sample, a BAM file including mapped and  
21 unmapped reads that spanned splice junctions was produced. Secondary alignment and multi-mapped  
22 reads were further removed using in-house scripts. Only uniquely mapped reads were retained for further  
23 analyses. Quality control metrics were performed using RseqQC using the m10 gene model provided.  
24 These steps include: number of reads after multiple-step filtering, ribosomal RNA reads depletion, and  
25 defining reads mapped to exons, UTRs, and intronic regions. Picard tool was implemented to refine the  
26 QC metrics (<http://broadinstitute.github.io/picard/>). Genecode annotation for mm10 (version M21) was  
27 used as reference alignment annotation and downstream quantification. Gene level expression was

1 calculated using HTseq version 0.9.1 using intersection-strict mode by gene (Anders et al., 2015). Counts  
2 were calculated based on protein-coding genes from the annotation file.

3

4 **Differential Expression.** Counts were normalized using counts per million reads (CPM). Genes with no  
5 reads in either *Mef2c*-Het or WT samples were removed. Differential expression analysis was performed  
6 in R using linear modeling as following:  $lm(\text{gene expression} \sim \text{Treatment})$   
7 Fitting this model, we estimated  $\log_2$  fold changes and P-values. P-values were adjusted for multiple  
8 comparisons using a Benjamini-Hochberg correction (FDR). Differentially expressed genes where  
9 consider for  $FDR < 0.05$ .

10

11 **Functional Enrichment.** The functional annotation of differentially expressed and co-expressed genes  
12 was performed using ToppGene (Chen et al., 2009). We used GO and KEGG databases. Pathways  
13 containing between 5 and 2000 genes were retained. A Benjamini-Hochberg FDR ( $P < 0.05$ ) was applied  
14 as a multiple comparisons adjustment.

15

16 **Gene set enrichment.** Gene set enrichment was performed using a Fisher's exact test in R with the  
17 following parameters: alternative = "greater", conf.level = 0.95. We reported Odds Ratios (OR) and  
18 Benjamini-Hochberg adjusted P-value (FDR).

19

20 **Availability of data and material.** The NCBI Gene Expression Omnibus (GEO) accession number will  
21 be made available upon publication.

22

23 **Code availability.** Custom R codes and data to support the analysis, visualizations, functional and gene  
24 set enrichments will be made available upon publication.

25

26 **Barrel Cortex Immunohistochemistry:** Mice were transcardially perfused with 1X PBS followed by 4%  
27 paraformaldehyde (PFA) in PBS. Brains were hemisected and cortex was isolated. Cortices were  
28 flattened between 2 glass slides and post-fixed in 4% PFA for >24 hours. Cortices were cryoprotected in

1 sucrose then sliced on a sliding stage microtome at 40  $\mu$ M. Slices were blocked with 5% normal donkey  
2 serum and 1% albumin from bovine serum (0.3% TritonX-100, 1X PBS) for 2 hours, incubated with  
3 primary antibody anti-vGLUT2 (Abcam ab79157; 1:500) for overnight, incubated with Cy3 conjugated  
4 secondary antibody, and dehydrated. Cover slips were mounted using DPX mountant (Sigma).

5  
6 **Electrophysiology:** All acute-slice electrophysiological experiments were performed in control and  
7 *Mef2c*-het mice at ages P30-P40. Acute coronal slices (300- $\mu$ m thickness) containing barrel cortex were  
8 prepared in a semi-frozen 300 mOsm dissection solution containing (in mM): 100.0 choline chloride, 2.5  
9 KCl, 1.25 Na<sub>2</sub>H<sub>2</sub>PO<sub>4</sub>, 25.0 NaHCO<sub>3</sub>, 25.0 D-glucose, 3.1 Na pyruvate, 9 Na ascorbate, 7.0 MgCl<sub>2</sub>, 0.5  
10 CaCl<sub>2</sub> and 5.0 kynurenic acid and was continually equilibrated with 95% O<sub>2</sub> and 5% CO<sub>2</sub> prior to and  
11 during the slicing procedure. Slices were transferred to a 315 mOsm normal artificial cerebrospinal fluid  
12 (ACSF) solution containing (in mM): 127 NaCl, 2.5 KCl, 1.20 Na<sub>2</sub>H<sub>2</sub>PO<sub>4</sub>, 24 NaHCO<sub>3</sub>, 11 D-glucose,  
13 1.20 MgCl<sub>2</sub>, and 2.40 CaCl<sub>2</sub>, 0.4 Na Ascorbate to recover at 37°C for 30 minutes, and then transferred  
14 to room temperature ACSF for an additional 30 minutes prior to recording.

15 Layer 2/3 (L2/3) pyramidal neurons (depth 30-100  $\mu$ m into the slice) of barrel cortex were visualized  
16 with infrared differential interference contrast optics (DIC/infrared optics) and identified by their location,  
17 apical dendrites, and burst spiking patterns in response to depolarizing current injection. Unless stated  
18 otherwise, all electrophysiological experiments were performed in whole cell voltage clamp mode at -70  
19 mV using borosilicate pipettes (4-6 M $\Omega$ ) made on NARISHIGE puller (NARISHIGE, PG10) from  
20 borosilicate tubing (Sutter Instruments) and filled by an internal solution containing (in mM): 120 K-  
21 Gluconate, 5 NaCl, 10 HEPES, 1.1 EGTA, 4 MgATP, 0.4 Na<sub>2</sub>GTP, 15 phosphocreatine, 2 MgCl<sub>2</sub>, and  
22 0.1 CaCl<sub>2</sub>.

23 All data (Recordings) were acquired and analyzed by amplifier AXOPATCH 200B (Axon Instruments),  
24 digitizer BNC2090 (X National instruments) and software AxoGraph v.1.7.0, Clampfit v 8.0 (pClamp,  
25 Molecular devices) and Mini Analysis Program v.6.0.9 (Synaptosoft). Data were filtered at 2 kHz by  
26 AXOPATCH 200B amplifier (Axon Instruments) and digitized at 10-20 kHz via AxoGraph v.1.7.0.

27 **Evoked postsynaptic currents:** The evoked postsynaptic responses of BC pyramidal neurons in  
28 L2/3 were elicited by field stimulation of excitatory afferents in L4 directly underneath the recorded cells

1 or in L2/3 of the adjustment barrel column at frequency of 0.05 Hz ( $0.05\text{ c}^{-1}$ ) - 3 stimulus in one second.  
2 The low-intensity pulses of stimulated current (25–100 mkA, 50–100 mks duration) were applied through  
3 a fine-tipped (~2 mkm), bipolar stimulating electrode made from the borosilicate theta glass capillary  
4 tubing.

5 AMPA-receptor-mediated excitatory postsynaptic currents (EPSCs) were recorded in presence of  
6 picrotoxin (100 mkM, Sigma Aldrich) to block GABA<sub>A</sub>Rs. Inhibitory postsynaptic currents (IPSCs)  
7 mediated by GABA<sub>A</sub> receptors were recorded in presence of DNQX (20 mkM, Tocris) to eliminate the  
8 current through AMPA-receptors.

9 For Paired-Pulse Ratio (PPR) measurements, two EPSC amplitudes were generated at -70mV with  
10 the inter-stimulus interval of 50 msec. The peak amplitude of the second EPSC (P2) was divided by the  
11 peak of the first amplitude (P1) to generate the PPR ratio (P2/P1).

12

13 **Miniature postsynaptic current:** Excitatory (E) and inhibitory (I) miniature postsynaptic currents  
14 (mPSCs) were recorded from L2/3 pyramidal neurons in voltage clamp mode at -70 mV. Pyramidal  
15 neurons were identified by their morphology parameters (pyramidal shape of soma, apical dendrite) and  
16 by bursting pattern of action potentials firing in response to depolarizing current injection. After  
17 identification, the normal ACSF was replaced by solution for mPSCs-records. For mEPSCs mediated by  
18 AMPARs, the extracellular bath solution (ACSF) contained 1  $\mu\text{M}$  tetrodotoxin (TTX, Sigma Aldrich) and  
19 100  $\mu\text{M}$  picrotoxin (Sigma Aldrich). mIPSCs were recorded using a high-chloride internal solution with a  
20 reversal potential at  $E_{\text{Cl}^-} = -15\text{ mV}$  for chloride containing (in mM): 79 (70) K-gluconate, 44 (75) KCl, 6(2)  
21 NaCl, 10(11) HEPES, 0.2 EGTA, 4(2) MgATP, 0.4 (0.2) Na2GTP, 2 (1) MgCl<sub>2</sub>, and 0.1 CaCl<sub>2</sub>. For record  
22 GABA<sub>A</sub>Rs-mediated mIPSC the extracellular bath solution (ACSF) contained 1  $\mu\text{M}$  TTX and 20  $\mu\text{M}$  DNQX  
23 (AMPA-receptor antagonist, Sigma-Aldrich). For recording AMPARs-mediated mEPSC the extracellular  
24 bath solution (ACSF) contained 1  $\mu\text{M}$  TTX and 100  $\mu\text{M}$  picrotoxin (GABA<sub>A</sub>Rs antagonist, Sigma-Aldrich).

25 At the beginning of each sweep, a depolarizing step (4 mV for 100 ms) was generated to monitor  
26 series (10-40 M $\Omega$ ) and input resistance (>400 M $\Omega$ ). Data were collected in a series of traces until >300  
27 events were recorded. Synaptic events were detected via custom parameters in MiniAnalysis software



1 (Synptosoft, Decatur, GA) and subsequently confirmed by observer. For each event, amplitude and  
2 frequency was measured and used to determine average mean.  
3  
4 **Dendritic Spine Analysis:** Dendritic spine labeling was done as previously described (Spencer et al.,  
5 2018) with some modifications. Briefly, mice were lightly transcardially perfused with 1.5% PFA in PBS  
6 and brains were post-fixed for 1 hour at 4°C in 1.5% PFA. Brains were sectioned at 200  $\mu\text{m}$  using a  
7 vibrating microtome (Leica VT100P). Tungsten particles (1.3  $\mu\text{m}$  diameter; Bio-Rad) were coated with  
8 lipophilic carbocyanine dye Dil (Life Technologies) and diolistically delivered into cortical regions using a  
9 Helios Gene Gun system (Bio-Rad) fitted with a polycarbonate filter (3.0  $\mu\text{m}$  pore size; BD Biosciences).  
10 After delivering the Dil-coated particles, slices were incubated in PBS at 4°C overnight to allow the dye  
11 to diffuse along the neuronal dendrites and axons, then post-fixed for 1 hour in 4% PFA before mounting  
12 with Prolong<sup>®</sup> Gold Antifade (Invitrogen) and imaging. Images of secondary and tertiary L2/3 apical (4-7  
13 segments per animal, 31 segments per group) and tertiary basal dendrites (3-7 segments per animal,  
14 WT=31 segments, het=26 segments) and L5 tertiary basal dendrites (3-5 segments per animal, WT=23  
15 segments, het=21 segments) in the barrel field were imaged with a Leica SP8 laser scanning confocal  
16 microscope equipped with HyD detectors for improved sensitivity. 50  $\mu\text{m}$  dendritic segments were imaged  
17 with a 63X oil immersion objective (1.4 NA) at 1024x512 frame size, 4.1x digital zoom, and 0.1  $\mu\text{m}$  Z-  
18 step size, generating a voxel size of 44 x 44 x 100 nm. Dil was excited using an OPAL 552 laser line with  
19 a pinhole of 0.8 Airy Units (AU). Laser power and gain were initially optimized, and then held relatively  
20 constant for the remainder of the experiment (i.e. laser power and gain were dynamically adjusted to  
21 avoid saturated voxels). Imaging parameters were chosen based off the Nyquist theorem as  
22 recommended by Huygens software (Scientific Volume Imaging, Hilversum, NL), which was used for  
23 deconvolution. Deconvolved images were imported into BitPlane Imaris (Version 9.1, Zurich, CH) for 3D  
24 reconstruction. The filament module was used to trace each dendrite, and the autopath tool was used to  
25 assign spines across each dendrite. Spine density per  $\mu\text{m}$  of dendrite and average spine head diameter  
26 of each dendritic segment were exported as variables.

27

1 **Tamoxifen Treatment of *Mef2c* *cHet*<sup>*Cx3Cr1*</sup> and *Cx3Cr1*<sup>*creER/+*</sup>; Ai14 Mice:** To induce recombination of  
2 the *Mef2c* floxed allele or Gt(ROSA)26Sor locus to express tdTomato in microglia, *Cx3Cr1*<sup>*creER/+*</sup>, *Mef2c*<sup>*fl/+*</sup>  
3 (experimental) and *Cx3Cr1*<sup>*creER/+*</sup>, *Mef2c*<sup>*+/+*</sup> (control) or *Cx3Cr1*<sup>*creER/+*</sup>; Ai14 pups were treated with  
4 tamoxifen via mother's milk (100 mg/kg of tamoxifen in 10% ethanol/90% sesame oil i.p. injection of dam)  
5 from post-natal day 1 through post-natal day 3. Starting at post-natal day 4, treated pups were then  
6 fostered by a lactating CD-1 female mouse until weaning.

7  
8 **Immunohistochemistry:** Mice were terminally anesthetized with ketamine/xylazine and transcardially  
9 perfused with PBS followed by 4% (w/v) paraformaldehyde (PFA). Brains were post-fixed for >24 hours  
10 at 4°C in 4% PFA then cryoprotected in 30% sucrose. Brains were coronally sectioned at 40-50 µm using  
11 a sliding microtome and stored in 1X PBS with 0.02% sodium azide. Anti-IBA1 IHC was performed based  
12 on manufacturer's protocol with modifications (Wako). Sections were washed 3 times for 10 minutes with  
13 1 X PBS with TritonX-100 (0.3%). Sections were then blocked with 1X PBS with 1% BSA and 0.3%  
14 TritonX-100 for 2 hours. Sections were immunostained with primary rabbit anti-IBA1 antibody (1:1000;  
15 Wako 019-19741) overnight at 4°C followed by Cy3 conjugated goat anti-rabbit secondary antibody  
16 (1:400 for 2 hours) or Alexa-Fluor-647 donkey anti-rabbit secondary antibody (1:400 for 2 hours).  
17 Sections were mounted with Prolong Gold with DAPI mountant (ThermoFisher).

18 Anti-GFP and anti-MEF2C IHC was performed as described in the following. Sections were washed 3  
19 times for 10 minutes with 1 X PBS with TritonX-100 (0.3%) and then blocked in 3% bovine serum albumin,  
20 3% normal donkey serum, 0.3% triton X-100, 0.2% tween-20 in 1X PBS. Sections were immunostained  
21 with primary chicken anti-GFP (1:1000; Aves GFP-1020) antibody and rabbit anti-MEF2A/MEF2C  
22 (1:300; abcam ab197070) antibody overnight at 4°C followed by Alexa Fluor-488 donkey anti-chicken  
23 (1:400) and Cy3 donkey anti-rabbit secondary antibody (1:200) for 1.5 hours. Sections were mounted  
24 with Prolong Gold with DAPI mountant (ThermoFisher).

25  
26 **Confocal Imaging and Image Analysis:** Iba1 stained sections were imaged on a Zeiss 880 confocal  
27 microscope at 20x objective. On average, 3 images were imaged per mouse/brain region. Images were  
28 deconvolved in AutoQuant (Bitplane) and analyzed in Imaris software (Bitplane). Imaris analysis began

1 with surface rendering of Iba1+ cells. The mean intensity of each cell, cell number, and cell soma volume  
2 were calculated in the Imaris software. The mean intensity of each cell by animal was entered into  
3 GraphPad Prism software to generate cumulative frequency distributions. Statistical testing was  
4 performed on this data via Kolmogorov–Smirnov test. Iba1+ cell numbers per field and cell soma volumes  
5 were entered into GraphPad Prism by genotype and brain region. Genotype-based differences in Iba1+  
6 cell numbers were assessed via unpaired two-tailed or nested t-tests.

7 Imaging of MEF2C expression in microglia was performed on a Zeiss 880 confocal microscope at 40x  
8 objective.

9 Imaging to confirm microglia-specific recombination was performed in *Cx3Cr1<sup>creER/+</sup>*; Ai14 mice that  
10 express tdTomato under the *Gt(ROSA)26Sor* locus. Stained sections were imaged on a Zeiss 880  
11 confocal microscope at 20x objective. Quantification was performed in Fiji by counting.

12

13 **Cytokine Antibody Array:** The cytokine antibody array was performed according to manufacturer's  
14 instructions except the membranes were blocked for 2 hours. A starting concentration of 450 µg of cortical  
15 brain tissue from *Mef2c*-Het and controls were used for each membrane.

16