Research Article

Genetic dissection of BDNF and TrkB expression in glial cells

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Abstract

The brain-derived neurotrophic factor (BDNF) and its high-affinity receptor tropomyosin-related kinase receptor B (TrkB) are widely expressed in the central nervous system. It is well documented that neurons express BDNF and full-length TrkB (TrkB.FL), and a lower level of truncated TrkB (TrkB.T). With conflicting results, glial cells also have been reported to express BDNF and TrkB. In the current study, we employed a more sensitive and reliable genetic method to characterize the expression of BDNF and TrkB in glial cells in the mouse brain. We utilized three Cre mouse strains in which Cre recombinase is expressed in the same cells as BDNF, TrkB.FL, or all TrkB isoforms, and crossed them to Cre-dependent EGFP reporter mice to label BDNF- or TrkB- expressing cells. We performed immunohistochemistry with glial cell markers to examine the expression of BDNF and TrkB in microglia, astrocytes, and oligodendrocytes. Surprisingly, we found no BDNF- or TrkB- expressing microglia in the brain and spinal cord. Consistent with previous studies, most astrocytes only express TrkB.T in the adult brain. Moreover, there are a small number of astrocytes and oligodendrocytes that express BDNF, the function of which is to be determined. We also found that oligodendrocyte precursor cells, but not

mature oligodendrocytes, express both TrkB.FL and TrkB.T in the adult brain. These results not only clarify the expression of BDNF and TrkB in glial cells, but also open opportunities to investigate previously unidentified roles of BDNF and TrkB in glial cells.

Keywords: BDNF, TrkB, Cre, glia, microglia, astrocyte, oligodendrocyte

1. Introduction

Brain-derived neurotrophic factor (BDNF) is a neurotrophin that is widely expressed in the central nervous system. BDNF signals via the tropomyosin-related kinase B (TrkB) receptor to regulate neuronal proliferation, differentiation and survival, and synaptic function [1]. Binding of BDNF to the full-length TrkB (TrkB.FL) receptor triggers dimerization of TrkB.FL and tyrosine autophosphorylation, leading to activation of three main downstream signaling cascades mediated by phospholipase C γ1 (PLCγ1), phosphoinositide 3-kinase, and RAS/RAF/mitogen-activated protein kinase, respectively [2]. Alternative splicing of *Ntrk2* exons produces the truncated TrkB (TrkB.T) isoforms that lack nearly all the intracellular domain including the tyrosine kinase motif [3]. TrkB.T can negatively regulate TrkB.FL signaling by forming a TrkB.FL-TrkB.T heterodimer and inhibiting the activation of TrkB.FL [4]. TrkB.T also functions to sequester and translocate BDNF, induce neurite outgrowth, and activate downstream protein kinase C and PLCγ via G-protein and inhibit Rho GTPase via dissociating Rho GDP dissociation inhibitor [4]. Because of the diverse roles of the BDNF-TrkB pathway, dysregulation of the BDNF-TrkB signaling has been implicated in various disease conditions, including cognitive impairment, obesity, neurodegenerative diseases, and cancer [5].

BDNF-TrkB signaling pathway has been studied extensively in neurons. It is well-documented that neurons express BDNF, TrkB.FL and a lower level of TrkB.T. Expression of BDNF and TrkB has also been reported in glial cells. BDNF expressed in microglia was suggested to be involved in neuropathic pain and motor-learning dependent synapse formation [6, 7].

53 However, other studies showed very low or no expression of BDNF in microglia [8]. Therefore, it
54 is debatable whether microglia express BDNF or not. It has been shown that astrocytes
55 predominantly express TrkB.T [1, 9]. Whether astrocytes also express TrkB.FL is not clear yet.
56 Some studies have shown TrkB.FL expression in astrocytes isolated from mouse brain [10, 11],
57 whereas other studies show no TrkB.FL expression in astrocytes [12, 13]. Astrocytes can
58 internalize proBDNF secreted by neurons, convert proBDNF to mature BDNF (mBDNF) and
59 release mBDNF for synaptic reuse [14, 15]. Astrocytes was shown to express BDNF *in vitro* which
60 regulates neuronal dendrite maturation [16], but whether astrocytes express BDNF *in vivo* under
61 physiological conditions is unknown. Oligodendrocytes have also been shown to express BDNF,
62 the reduction of which impairs presynaptic vesicular exocytosis [17]. Oligodendroglial expression
63 of TrkB is important for oligodendrocyte myelination and oligodendrocyte precursor cell (OPC)
64 proliferation [18-20]. But it has not been characterized which TrkB isoform oligodendrocytes
65 express and what percentage of oligodendrocytes express BDNF or TrkB.

66 To fill these knowledge gaps, in the current study, we employed a genetic method to label
67 and characterize the expression of BDNF and TrkB in glial cells, including microglia, astrocytes
68 and oligodendrocytes. We crossed the *Bdnf*$^{2A\text{-}Cre/+}$ [21], *Ntrk2*$^{2A\text{-}Cre/+}$, and *Ntrk2*$^{CreER/+}$ [22] mouse
69 strains to the Cre-dependent B6;129S4-Gt(*ROSA*)*26Sor*$^{tm9(EGFP/Rpl10a)Amc}$/J (EGFP-L10a) reporter
70 mice [23] to generate double heterozygous mice in which cells expressing BDNF or TrkB were
71 labeled by EGFP. We then performed immunofluorescence staining of cell type markers with the
72 brain sections from these mice. Colocalization of EGFP and cell type markers was analyzed to
73 determine the expression of BDNF and TrkB in glial cells. There are several advantages of this
74 method: (1) high sensitivity because a very small amount of Cre expression can induce EGFP
75 expression in the reporter mice; (2) no involvement of BDNF and TrkB antibodies which have
76 non-specific immunoreactivity; (3) no need to isolate glia from the mouse brain, which involves
77 potential contamination of other cell types; (4) being able to observe expression of BDNF and
78 TrkB *in vivo* at the level of single cells.

79 Our results show that homeostatic and activated microglia do not express either BDNF or
80 TrkB. Astrocytes predominantly express TrkB.T, and a small astrocyte population express
81 TrkB.FL and BDNF in the adult mice. A subset of oligodendrocytes express BDNF in the
82 hippocampal CA1, as well as TrkB.T and TrkB.FL.
83

84 **2. Materials and Methods**

85 2.1 Animals

86 Mice of the EGFP-L10a/+ (B6;129S4-Gt(*ROSA*)*26Sor*$^{tm9(EGFP/Rpl10a)Amc}$/J; stock #024750)
87 and the *Bdnf*$^{2A\text{-}Cre/+}$ (B6.FVB-*Bdnf*$^{em1)cre_Zak}$/J; stock #030189) strains were obtained from the
88 Jackson Laboratory. The *Ntrk2*$^{CreER/+}$ mouse strain was generously provided by Dr. David Ginty
89 at Harvard Medical School [22]. *Ntrk2*$^{2A\text{-}Cre/+}$, *Ntrk2*$^{CreER/+}$, and *Bdnf*$^{2A\text{-}Cre/+}$mice were crossed to
90 EGFP-L10a/+ mice to generate *Ntrk2*$^{2A\text{-}Cre/+}$;EGFP-L10a/+, *Ntrk2*$^{CreER/+}$;EGFP-L10a/+ and *Bdnf*$^{2A\text{-}}$
91 $^{Cre/+}$;EGFPL10a/+ mice. All experiments were performed in accordance with relevant guidelines
92 and regulations regarding the use of experimental animals. The Animal Care and Use Committees
93 at the UF Scripps approved all animal procedures used in this study.
94

95 2.2 Generation of *Ntrk2*$^{2A\text{-}Cre/+}$ mice

96 We generated a *Ntrk2*$^{2A\text{-}Cre}$ mouse allele in which the DNA sequence encoding P2A-Cre
97 recombinase was inserted immediately before the stop codon for TrkB.FL at the *Ntrk2* locus using
98 the CRISPR/Cas9 technique. sgRNA (5'-AGTCAAGAGGTTCGTCGTGT-3') was designed using
99 the CRISPR tool (http://crispr.mit.edu) to minimize potential off-target effects. The DNA donor
100 plasmid contains two homology arms with 840-bp flanking the P2A-Cre sequence, which were
101 cloned into BamHI digested pBluescript II KS (-) vector using the Gibson assembly method (NEB,
102 E5510). To block further Cas9 targeting and recutting after undergoing homology-direct repair,
103 we introduced a silent mutation into the PAM motif of the sgRNA located within the 3' homology
104 arm in the donor plasmid by using Q5 Site-Directed Mutagenesis kit (NEB, E0054). The donor

105 plasmid was confirmed with DNA sequencing. Microinjection of a mixture of sgRNA, donor DNA,
106 and Cas9 protein was performed by the Genomic Modification Facility at Scripps Research.
107 Zygotes were cultured to the blastocyst stage *in vitro*. Genomic DNA from blastocysts was
108 extracted and PCR screen was performed to select blastocysts with successful homologous
109 recombination. Positive blastocysts were transferred into the oviduct of pseudo-pregnant females
110 to produce founder mice. Two out of twenty-eight pups were positive for the knockin, confirmed
111 by the genomic DNA PCR using the following primers: CCTCCTGGTGAGCAAACGAT (forward)
112 and GACATAGGGCCGGGATTCTC (reverse) for PCR across the 5' homology arm, and
113 CACCTCCATGCCTGTGTTTT (forward) and GGTTCTTGCGAACCTCATCA (reverse) for PCR
114 across the 3' homology arm. The founder mice were crossed to C57BL/6J to produce mice with
115 germline transmission. Cre expression in these lines were confirmed by *in situ* hybridization.
116
117 2.3 In situ hybridization
118 Freshly dissected mouse brains were quickly frozen in a 2-methylbutane (Fisher Scientific
119 #O3551-4) dry ice bath. Subsequently, 14-μm thick cryostat sections were collected onto
120 Superfrost Plus slides (Fisherbrand #12-550-15) and used for in situ hybridization. Fluorescence
121 in situ hybridization was performed using the RNAscope Multiplex Fluorescent Reagent Kit v2
122 (#323100, Advanced Cell diagnostics). Brain sections were fixed with pre-chilled 10% formalin at
123 4°C for 15 min, and dehydrated with 50%, 75% and 100% ethanol. Sections were air-dried for 20
124 min and followed by v2Hydrogen peroxide for 10 min and pretreated with protease IV for 30 min,
125 washed 2 times in PBS and incubated with the target probes (Ntrk2-C3, #423611-C3; Cre-C2,
126 #31228-C2) for 2 hours at 40°C. Signals were amplified by subsequent incubation with v2Amp1
127 for 30 min, v2Amp2 for 30 min, v2Amp3 for 15 min, v2C2/C3HPR for 15 min and followed by TSA
128 Plus Fluorescein/Cyanine5 (NEL754001KT, Akoya Biosciences) for 30 min at 40°C. Slides were
129 counterstained with DAPI, and images were acquired using a Nikon C2+ confocal microscope.
130

131 2.4 Immunohistochemistry

132 Mice were perfused with phosphate-buffered saline (PBS) and then 4% paraformaldehyde
133 (PFA) in PBS. The brain was dissected and postfixed overnight in 4% PFA-PBS. Mouse brains
134 were soaked in 30% sucrose-PBS for cryoprotection. Brains were sectioned into 40-μm-thick
135 slices using a sliding microtome (Leica). Brain sections were blocked in 0.3% Triton X-100 and
136 10% normal horse serum before overnight incubation in primary antibody at 4 °C. The following
137 primary antibodies were used: guinea pig anti-Iba1(1:500; Synaptic System #234308), goat anti-
138 SOX9 (1:2000; R&D Systems #AF3075), mouse anti-GFAP (1:500; Cell Signaling Technology
139 #3670S), mouse anti-Olig2 (1:250; Millipore #MABN50), rabbit anti-ASPA (1:1500; GeneTex
140 #GTX113389), chicken anti-GFP (1:2000; abcam #13970), rabbit anti-DsRed (1:1000; TaKaRa
141 #632496). The brain sections were washed three times with PBS before incubation in secondary
142 antibody for 1 hour at room temperature. The brain sections were then counterstained with DAPI,
143 mounted onto microscope slides, and covered with mounting media.

144

145 2.5 LPS injection

146 LPS (Millipore #L6529) was dissolved in sterile saline to a final concentration of 0.1 μg/μL.
147 The mice were given daily saline or LPS injection at a dose of 500 μg/kg body weight for seven
148 consecutive days. The mice were harvested the next day after the last treatment.

149

150 2.6 Tamoxifen injection

151 Tamoxifen (Sigma #T5648-5G) was dissolved in 100% ethanol to a concentration of 20
152 mg/ml. An equal volume of corn oil was added to the tamoxifen solution. The corn oil/ethanol
153 mixture was vortexed, centrifuged for 10 mins at 13,000 rpm, and then placed in a vacuum
154 centrifuge for 30 mins to evaporate ethanol. Tamoxifen was given to *Ntrk2*$^{CreER/+}$;EGFP-L10a/+
155 mice at the age of 6 weeks through daily i.p. injection at a dose of 150 mg/kg body weight for 10

156 consecutive days to induce translocation of the Cre-ER fusion protein to the nucleus. The mice
157 were given three weeks to recover before they were harvested.
158
159 2.7 Plasmids and viruses
160 pAAV8-EF1a-Nuc-flox(mCherry)-EGFP was obtained from Addgene (plasmid #112677, a
161 gift from Brandon Harvey [24]). For higher expression in glial cells, the EF1a promoter was
162 replaced by the CAG promoter from pAAV-CAG-mNeonGreen (Addgene plasmid #99134, a gift
163 from Viviana Gradinaru [25]). pAAV8-EF1a-Nuc-flox(mCherry)-EGFP was digested with AgeI and
164 BamHI, and the CAG promoter fragment was amplified by PCR (forward primer: GAAT
165 ACCGGTCGTTACATAACTTACGGTAAATG; reverse primer:
166 GAATGGATCCCGCCCGCCGCGCGCTT). PCR product was digested with AgeI and BamHI and
167 ligated into the backbone of pAAV8-EF1a-Nuc-flox(mCherry)-EGFP. The resulting pAAV8-CAG-
168 Nuc-flox(mCherry)-EGFP plasmid was confirmed by sequencing and used to package AAV
169 viruses. AAV viruses were purified with TaKaRa AAVpro Purification Kit Maxi (#6666).
170
171 2.8 Stereotaxic injection of adeno-associated virus
172 Mice were anesthetized using isoflurane and securely positioned on a stereotaxic holder
173 (David Kopf, Model 940). A small incision was made to expose the skull, and subsequently a
174 small hole was drilled on the skull above an injection site. For injection, a Nanofil 33-gauge needle
175 (World Precision Instruments, #NF33BV-2) was slowly inserted into the CA1 region (AP: -1.60
176 mm; ML: ± 0.80 mm; DV: −1.80 mm). AAV8-CAG-Nuc-flox(mCherry)-EGFP (150 nl at 3.12E+12
177 vg/mL) was then injected at a rate of 30 nl/min using a micro syringe pump (World Precision
178 Instruments, SYS-MICRO4). The needle was then withdrawn after staying in the same position
179 for 5 min. Following the injection, mice received Loxicom (5 mg/kg) for analgesia and were
180 returned to their home cages.
181

182 2.9 Imaging and Cell number quantification

183 Fluorescent images were captured using a Nikon C2+ confocal microscope. Cell numbers
184 were quantified using the cell counter plugin in FIJI software.

185

186 **3. Results**

187 **3.1 Homeostatic and activated microglia in the cortex, hippocampus and spinal cord do**
188 **not express BDNF**

189 *Bdnf*$^{2A\text{-}Cre/+}$ mice that express Cre recombinase in BDNF-expressing cells were crossed to
190 Cre-dependent EGFPL10a/+ reporter mice to generate *Bdnf*$^{2A\text{-}Cre/+}$;EGFPL10a/+ mice. Cre-
191 mediated excision of *loxP*-flanked STOP sequence results in constitutive expression of EGFP
192 localized in the nucleus and cytoplasm of Cre-expressing cells. EGFP is thus expressed in cells
193 that express BDNF at any time during the lifespan of a *Bdnf*$^{2A\text{-}Cre/+}$;EGFP-L10a/+ mouse. We first
194 used NeuN as a marker to label neurons. As expected, most neurons in the cortex and
195 hippocampal CA1 express BDNF (Fig. 1A-F).

196 Next, we wanted to know whether microglia express BDNF. For this, we used Iba1
197 antibody to label microglia in *Bdnf*$^{2A\text{-}Cre/+}$;EGFP-L10a/+ mice. Surprisingly, we did not observe
198 EGFP expression in nuclei of Iba1^{+} microglia in the somatomotor cortex (MO), hippocampal CA1
199 stratum radiatum (SR), or the spinal cord of 4-month-old *Bdnf*$^{2A\text{-}Cre/+}$;EGFPL10a/+ mice, indicating
200 that homeostatic microglia do not express BDNF under physiological conditions (Fig. 1G-O).
201 However, we did observe EGFP puncta in some microglial cell bodies and processes, outside
202 microglial nuclei (Fig. 1P-S). Immunostaining of CD68, a microglial lysosomal marker, showed
203 that the EGFP puncta are always colocalized with CD68 (Fig. 1P-S). This indicates that these
204 EGFP signals in microglia come from BDNF-expressing cells engulfed by microglia and are not
205 from BDNF expression in microglia.

206

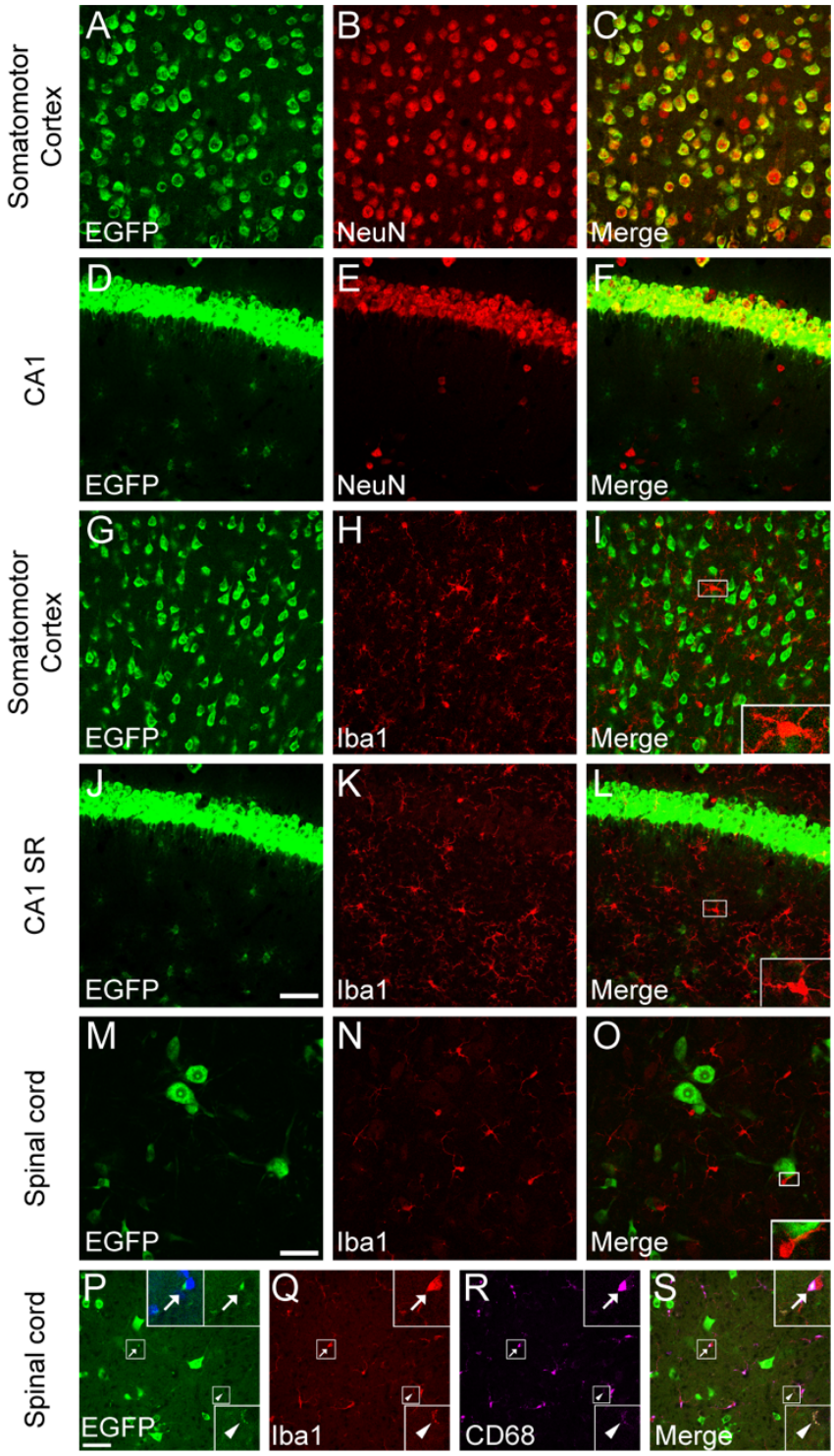


Figure 1. Homeostatic microglia do not express BDNF. (**A-F**) BDNF expression in NeuN$^+$ neurons in the MO (A-C) and CA1 (D-F). (**G-O**) Homeostatic microglia do not express BDNF in the MO (G-I), CA1 (J-L), and spinal cord (M-O). (**P-S**) Colocalization of EGFP puncta signals with CD68$^+$ microglia lysosomes. Arrows denote EGFP signal inside microglial cytoplasm. Arrowheads denote EGFP signal inside microglial processes. Scale bar = 50 μm. n = 2 mice.

Microglia can exist in homeostatic or activated states. To investigate whether activated microglia express BDNF under pathological conditions, we injected lipopolysaccharide (LPS) to induce microglial activation in adult *Bdnf*$^{2A\text{-}Cre/+}$;EGFPL10a/+ mice. CD68 is used as a marker for microglia activation, as activated microglia express a higher level of CD68. LPS treatment resulted in a significant increase in CD68 intensity per microglia (Fig. 2). We did not observe EGFP signal in Iba1^{+} microglial nuclei in LPS-injected mice, suggesting that activated microglia also do not express BDNF (Fig. 2E-H). Therefore, our results suggest that both homeostatic and activated microglia do not express BDNF.

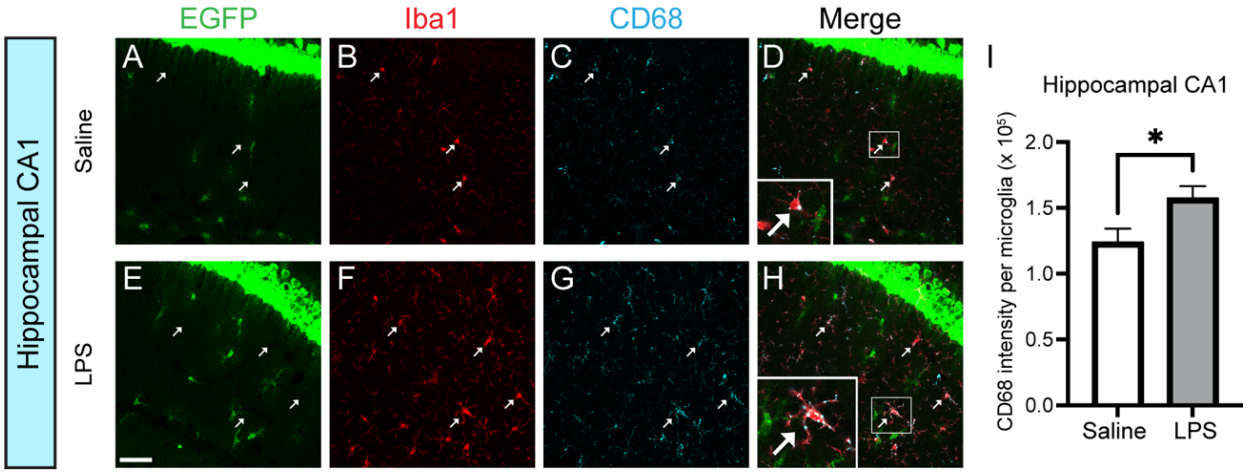


Figure 2. Activated microglia do not express BDNF. (**A-H**) Immunostaining of Iba1 and CD68 in the hippocampus of *Bdnf*$^{2A\text{-}Cre/+}$;EGFPL10a/+ mice injected with saline (A-D) or LPS (E-H). Arrows indicate colocalization of Iba1 and CD68, and no EGFP inside microglia. Scale bar = 50 μm. (**I**) Quantification of CD68 intensity per microglia. n = 1 35 microglia from one saline mouse and 50 microglia from 2 LPS mice . *p = 0.0204 by two-sided *t*-test. Data are shown as mean ± s.e.m.

3.2 Some astrocytes express BDNF in the cortex and hippocampus

Whether astrocytes express BDNF *in vivo* under physiological conditions is unknown. To answer this question, we labeled astrocytes in *Bdnf*$^{2A\text{-}Cre/+}$;EGFPL10a/+ mice using SOX9 antibody for the somatosensory cortex (SS) and GFAP antibody for the hippocampus. We observed that a small portion of astrocytes (2.6% in the SS and 18.3% in the CA1 SR) are EGFP^{+}

in the *Bdnf*$^{2A\text{-}Cre/+}$;EGFPL10a/+ mice, indicating that these cells express BDNF at some point during the lifetime of the mice (Fig. 3A-H). The EGFP signals in these mice are cumulative, because once the EGFP expression in a cell is turned on by Cre, it is never turned off even if the cell stops to express Cre. Therefore, we cannot know when BDNF is expressed in these mice.

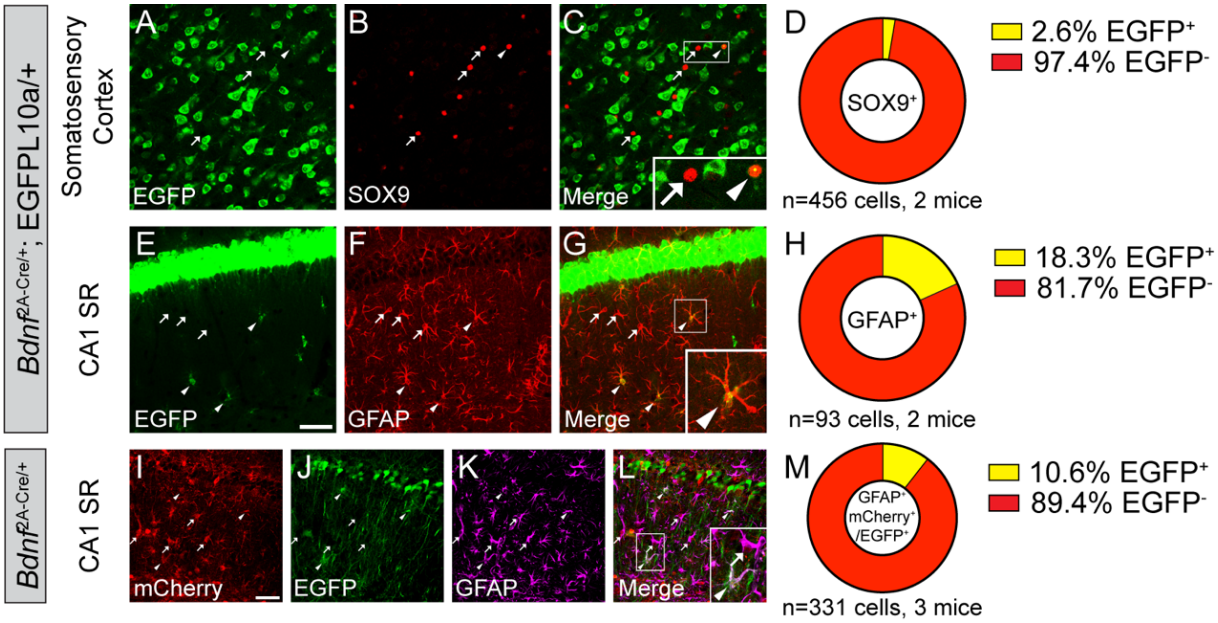


Figure 3. A small subset of astrocytes express BDNF in the hippocampus. (**A-H**) Immunostaining of SOX9 (A-C) and GFAP (E-G) in the SS (A-C) and CA1 SR (E-G) of *Bdnf*$^{2A\text{-}Cre/+}$;EGFPL10a/+ mice, and (D, H) quantification of colocalization. (**I-M**) Immunostaining of GFAP in the CA1 SR of adult *Bdnf*$^{2A\text{-}Cre/+}$ mice injected with the mCherry-to-EGFP color switch AAV, and (M) quantification of colocalization. Scale bar = 50 μm. Arrowheads denote EGFP-expressing astrocytes. Arrows denote astrocytes that do not express EGFP.

To test whether astrocytes in adult mice still express BDNF, we injected a Cre-dependent mCherry-to-EGFP color switch adeno-associated virus (AAV), AAV8-CAG-Nuc-flox(mCherry)-EGFP, into the hippocampal CA1 of 2-month-old *Bdnf*$^{2A\text{-}Cre/+}$ mice. In these mice, transduced Cre-negative cells will express mCherry, while transduced Cre-positive cells will express EGFP. Around 87% astrocytes were transduced by the AAV, and 10.6% of the transduced astrocytes express BDNF in adult mice (Fig. 3I-M). This suggests that a small portion of astrocytes in the CA1 SR still express BDNF in adult mice. Because this number is smaller than the cumulative

250 18.3% in *Bdnf*$^{2A\text{-}Cre/+}$;EGFPL10a/+ mice, there could be two possibilities: (1) some astrocytes only
251 express BDNF early during development and stop BDNF expression in adults; (2) at each different
252 time point, there is a different small subset of astrocytes that express BDNF, and that accumulates
253 to the 18.3% seen in *Bdnf*$^{2A\text{-}Cre/+}$;EGFPL10a/+ mice. To figure out which possibility is true, future
254 studies can investigate BDNF expression at developmental stages.
255

256 **3.3 A small portion of oligodendrocytes express BDNF in the hippocampal CA1 SR**

257 Next, we examined BDNF expression in Olig2^{+} oligodendrocytes. We found that most
258 Olig2^{+} oligodendrocytes in the SS do not express BDNF, and 4.4% of Olig2^{+} oligodendrocytes in
259 the CA1 SR express BDNF at some point during the lifespan of *Bdnf*$^{2A\text{-}Cre/+}$;EGFPL10a/+ mice
260 (Fig. 4A-H). To find out whether these oligodendrocytes express BDNF in adult mice, we injected
261 the mCherry-to-EGFP color switch virus to the hippocampus of *Bdnf*$^{2A\text{-}Cre/+}$ mice and examined
262 colocalization of Olig2 with mCherry and EGFP. Out of the transduced Olig2^{+} cells, 14.5% express
263 BDNF in adult mice (Fig. 4I-M). It is unexpected that the percentage of BDNF-expressing
264 oligodendrocytes in adult is higher than that of cumulative expression in *Bdnf*$^{2A\text{-}Cre/+}$;EGFPL10a/+
265 mice. One possible explanation is that AAV injection might cause tissue damage or myelin
266 damage, which induced the increase in BDNF expression in oligodendrocytes.

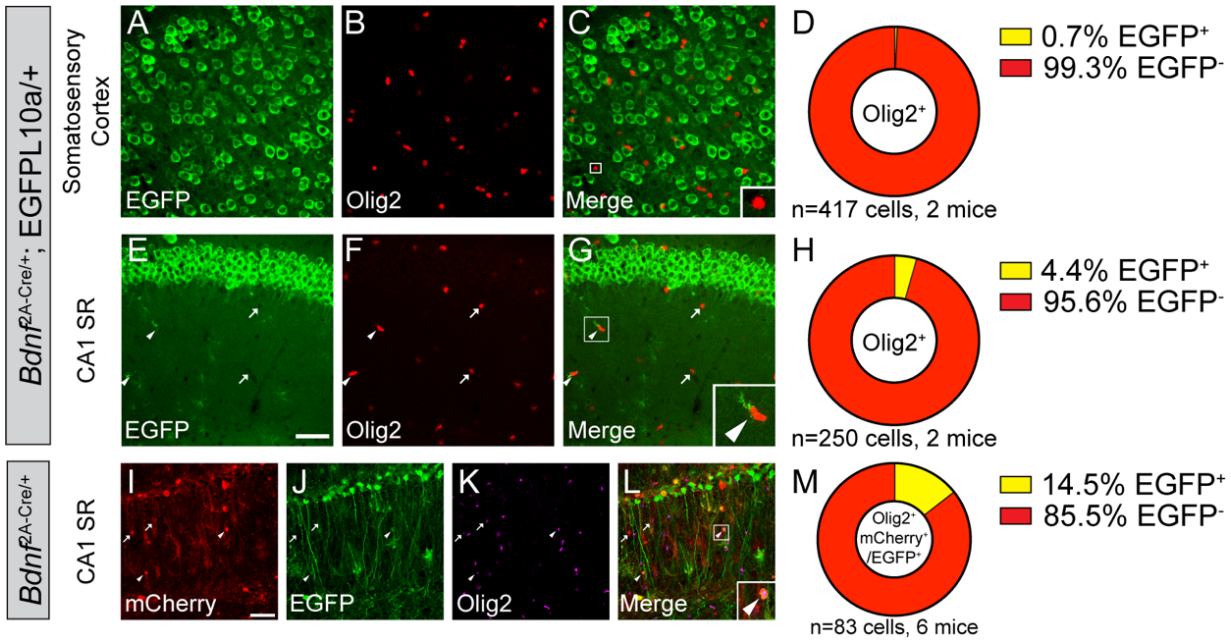


Figure 4. A small subset of oligodendrocytes express BDNF in the hippocampus. (**A-H**) Immunostaining of Olig2 in the SS (A-C) and CA1 SR (E-G) of adult *Bdnf*$^{2A\text{-}Cre/+}$;EGFPL10a/+ mice, and (D,H) quantification of colocalization. (**I-M**) Immunostaining of Olig2 in the CA1 SR of adult *Bdnf*$^{2A\text{-}Cre/+}$ mice injected with the mCherry-to-EGFP color switch AAV, and (M) quantification of colocalization. Scale bar = 50 μm. Arrowheads denote EGFP-expressing oligodendrocytes. Arrows denote oligodendrocytes that do not express EGFP.

3.4 Generation of *Ntrk2*$^{2A\text{-}Cre}$ allele.

To label cells that express TrkB.FL, we generated the *Ntrk2*$^{2A\text{-}Cre/+}$ mouse strain which express Cre recombinase in TrkB.FL-expressing cells. We employed the CRISPR/Cas9 technology to insert the P2A-Cre sequence into the *Ntrk2* locus immediately before the stop codon for the TrkB.FL (Fig. 5A). Mice with the insertion were identified using genomic DNA PCR (Fig. 5B). There was a high colocalization between *Cre* mRNA and *Ntrk2* mRNA for TrkB-FL in the cortex of *Ntrk2*$^{2A\text{-}Cre/+}$ mice (Fig. 5C-D), validating the allele.

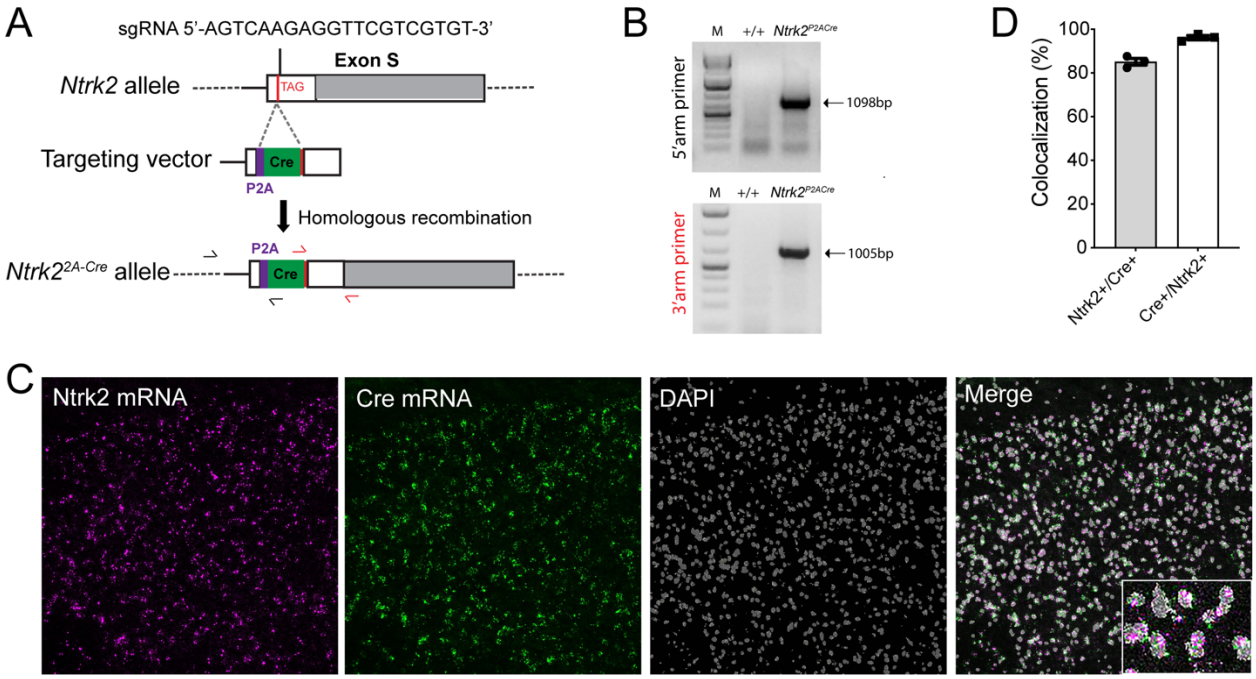
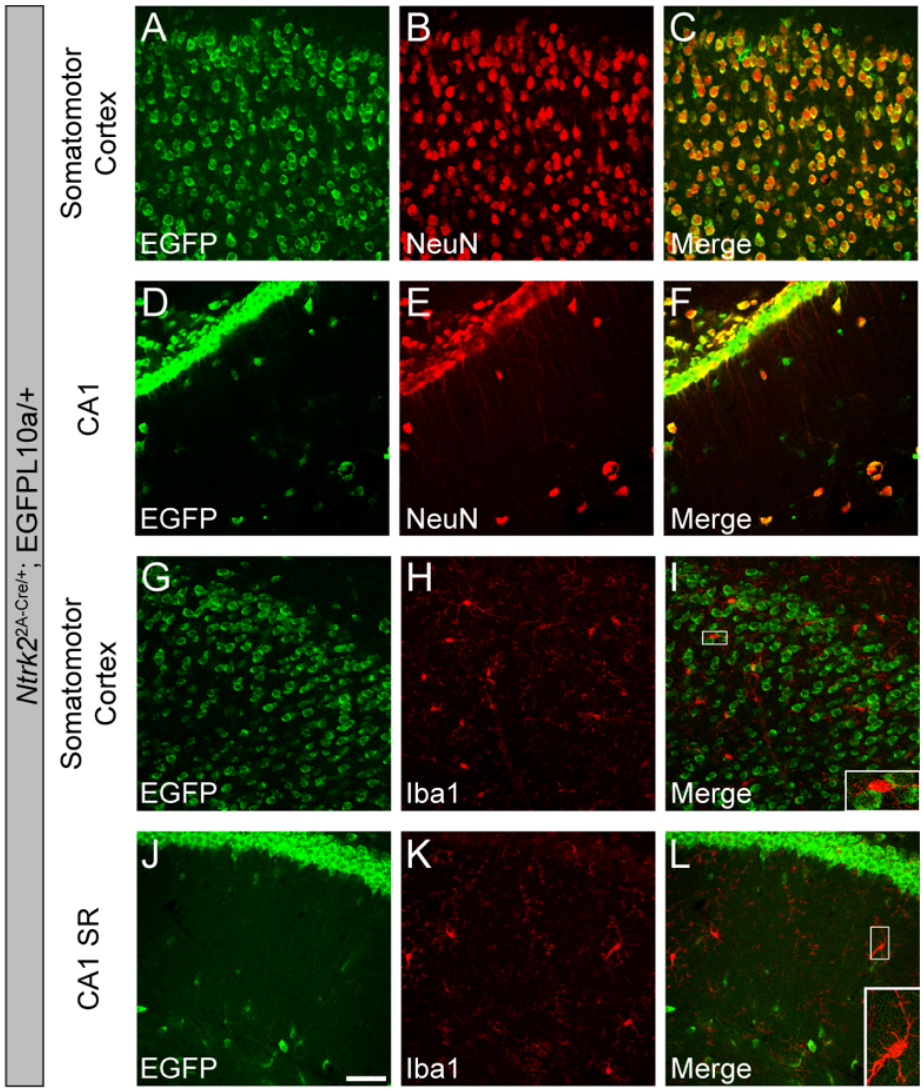


Figure 5. Generation of *Ntrk2*$^{2A\text{-}Cre}$ mice. (**A**) Strategy for insertion of the P2A-Cre sequence into the *Ntrk2* locus. (**B**) Genomic DNA PCR to identify *Ntrk2*$^{2A\text{-}Cre/+}$ mice. One primer is located outside each of the two homology arms in the targeting vector. (**C, D**) In situ hybridization of the cortex.

3.5 Microglia in the cortex, hippocampus or spinal cord do not express TrkB.FL or TrkB.T

To study TrkB.FL expression in glial cells, we crossed *Ntrk2*$^{2A\text{-}Cre/+}$ mice that express Cre in TrkB.FL-expressing cells to the Cre-dependent EGFP-L10a/+ reporter mice to generate *Ntrk2*$^{2A\text{-}Cre/+}$;EGFP-L10a/+ mice in which TrkB.FL expression is genetically labeled by EGFP. As expected, all neurons in the MO and hippocampal CA1 express TrkB.FL (Fig. 6A-F). Next, we examined whether microglia express TrkB.FL. No colocalization of EGFP and Iba1 was found in the MO or hippocampal CA1 SR of 4-month-old *Ntrk2*$^{2A\text{-}Cre/+}$;EGFP-L10a/+ mice, indicating that microglia in these regions do not express TrkB.FL any time up to the time when the mice were perfused (Fig. 6G-L).



295

296 **Figure 6. Microglia do not express TrkB.FL.** (**A-F**) Colocalization of NeuN^{+} neurons and EGFP in the MO and CA1

297 of *Ntrk2*$^{2A\text{-}Cre/+}$;EGFP-L10a/+ mice. (**G-L**) Homeostatic microglia do not express EGFP in the MO and CA1 SR of *Ntrk2*$^{2A\text{-}Cre/+}$;EGFP-L10a/+ mice. Scale bar = 50 μm. n = 2 mice.

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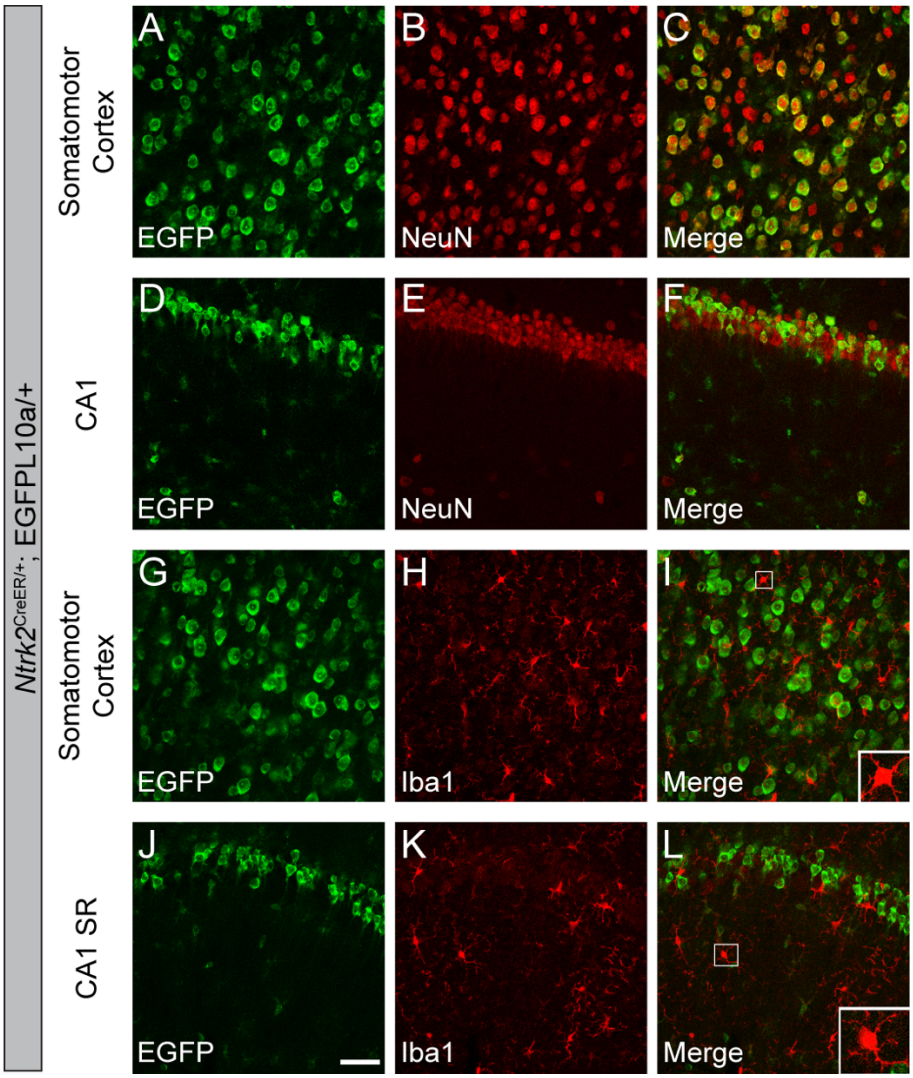


Figure 7. Microglia do not express either TrkB.FL or TrkB.T in adult mice. (**A-F**) Colocalization of NeuN^{+} neurons and EGFP in the MO and CA1 of *Ntrk2*$^{CreER/+}$;EGFP-L10a/+ mice. (**G-L**) Homeostatic microglia are not labeled by EGFP in the MO and CA1 SR of *Ntrk2*$^{CreER/+}$;EGFP-L10a/+ mice. Scale bar = 50 μm. n = 2 mice.

To see whether microglia express TrkB.T, we utilized *Ntrk2*$^{CreER/+}$ mice that express Cre-ER under the *Ntrk2* promoter. We crossed *Ntrk2*$^{CreER/+}$ mice to Cre-dependent EGFP-L10a/+ reporter mice to generate *Ntrk2*$^{CreER/+}$;EGFP-L10a/+ mice, in which cells expressing either TrkB.FL or TrkB.T are genetically labeled by EGFP upon tamoxifen exposure. As expected, neurons in the cortex and hippocampal CA1 are labeled by EGFP due to their expression of TrkB.FL and TrkB.T. However, only a subset of neurons were labeled by EGFP (Fig. 7A-F). Because almost all neurons should express TrkB.FL in adult mice (Fig. 6A-F), this suggests that

311 the Cre induction by tamoxifen was lower than 100%. Our data showed no colocalization of EGFP
312 and Iba1 in either MO or CA1 SR region (Fig. 7G-L). This means that Iba1^{+} microglia do not
313 express either TrkB.FL or TrkB.T in adult mice.
314
315 **3.5 Astrocytes predominantly express TrkB.T and a small subset of astrocytes express**
316 **TrkB.FL**
317 Astrocytes have been shown to predominantly express TrkB.T; however, whether
318 astrocytes also express TrkB.FL is not as clear. To investigate the expression of TrkB.FL in
319 astrocytes, we quantified colocalization of EGFP and astrocyte markers, SOX9 and GFAP, in
320 *Ntrk2*$^{2A\text{-}Cre/+}$;EGFP-L10a/+ mice. Our analyses showed that a small portion of astrocytes (16.8%
321 in SS and 18.0% in hippocampal CA1 SR) express TrkB.FL at some point during the lifespan of
322 the mice (Fig. 8A-H). To test whether astrocytes in adult mice express TrkB.FL, we injected the
323 Cre-dependent mCherry-to-EGFP color switch AAV to the hippocampus of 2-month-old *Ntrk2*$^{2A\text{-}}$
324 $^{Cre/+}$ mice [24]. 92.7% of astrocytes were transduced by the AAV virus, and only 3.6% of the
325 transduced cells express Cre (Fig. 8I-M). These results suggest that only a very small subset of
326 astrocytes express TrkB.FL in adult mice. Because this number is smaller than the 18.0% seen
327 in *Ntrk2*$^{2A\text{-}Cre/+}$;EGFP-L10a/+ mice. The two possibilities discussed before can also apply here: (1)
328 A small subset of astrocytes express TrkB.FL only early during development and stop the
329 expression in adult mice; (2) at each different time point, there is a different small subset of
330 astrocytes that express TrkB.FL, and that accumulates to the 18% seen in *Ntrk2*$^{2A\text{-}Cre/+}$;EGFP-
331 L10a/+ mice.
332

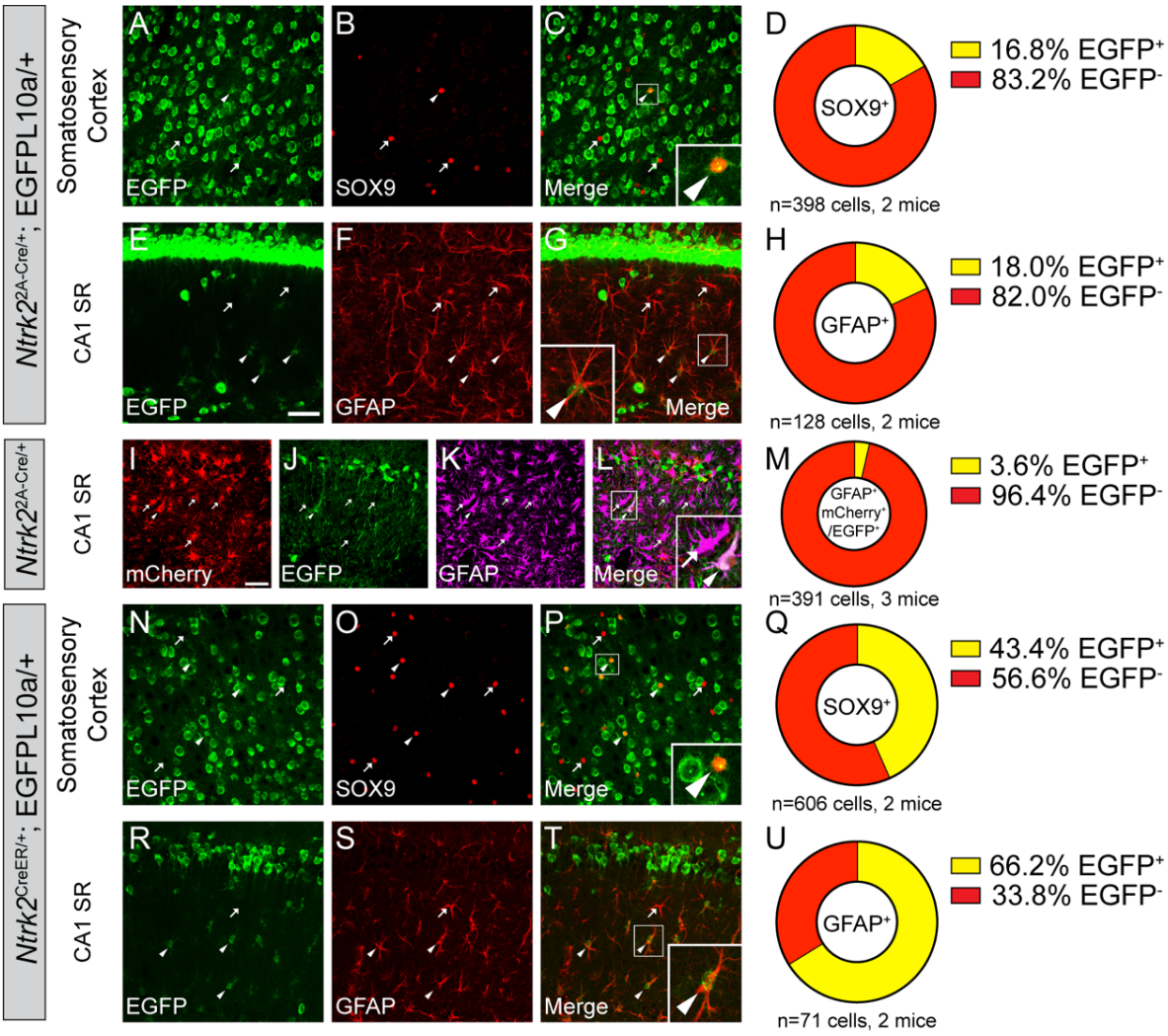


Figure 8. Astrocytes predominantly express TrkB.T in adult mice. (**A-G**) Immunostaining of SOX9 (A-C) or GFAP (D-G) in the SS (A-C) and CA1 SR (E-G) of *Ntrk2*$^{2A\text{-}Cre/+}$;EGFP-L10a/+ mice, and (D,H) quantification of colocalization. (**I-M**) Immunostaining of GFAP in the CA1 SR of adult *Ntrk2*$^{2A\text{-}Cre/+}$ mice injected with the mCherry-to-EGFP color switch AAV, and the quantification of the colocalization of GFAP and EGFP. (**N-U**) Immunostaining of SOX9 (A-C) or GFAP (D-G) in the SS (A-C) and CA1 SR (E-G) of *Ntrk2*$^{CreER/+}$;EGFP-L10a/+ mice, and the quantification of colocalization. Scale bar = 50 µm. Arrowheads denote EGFP-expressing astrocytes. Arrows denote astrocytes that do not express EGFP.

Next, we examined expression of TrkB.T using *Ntrk2*$^{CreER/+}$;EGFP-L10a/+ mice where cells expressing either TRKB.FL or TrkB.T are labeled by EGFP. Tamoxifen was given at the age of 6 weeks to induce Cre recombination. We found that 43.4% astrocytes express either TrkB.FL or

345 TrkB.T in the SS, and 66.2% in the CA1 SR (Fig. 8N-U). Because we showed that most astrocytes
346 do not express TrkB.FL in the CA1 SR of adult mice (Fig. 8I-M), this means most of the EGFP^{+}
347 astrocytes in the *Ntrk2*$^{CreER/+}$;EGFP-L10a/+ mice express TrkB.T only. Due to the incomplete Cre
348 induction described above, the actual percentage of astrocytes that express TrkB in
349 *Ntrk2*$^{CreER/+}$;EGFP-L10a/+ mice could be higher than what we show.
350

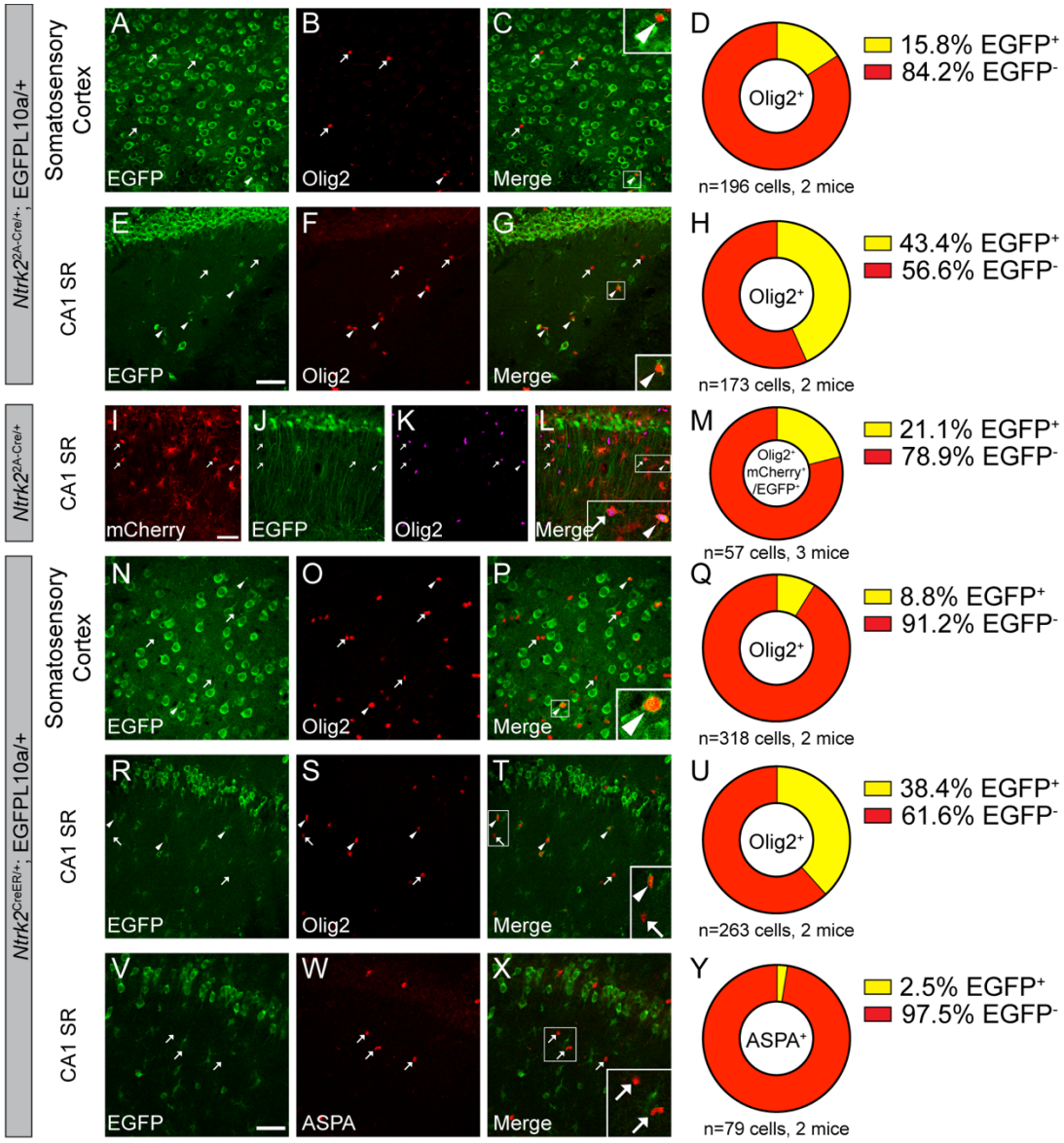
351 **3.6 Oligodendrocytes express TrkB.FL and TrkB.T in adult brains**

352 Lastly, we investigated TrkB expression in oligodendrocytes. Colocalization of
353 oligodendrocyte marker Olig2 and EGFP in *Ntrk2*$^{2A\text{-}Cre/+}$;EGFP-L10a/+ mice suggests that a
354 subset of oligodendrocytes (15.8% in SS and 43.4% in CA1 SR) express TrkB.FL at some point
355 during their lifespan (Fig. 9A-H). To examine whether oligodendrocytes express TrkB.FL in adult
356 mice, the Cre-dependent mCherry-to-EGFP color switch AAV was injected to the hippocampus
357 of adult *Ntrk2*$^{2A\text{-}Cre/+}$ mice. The transduction rate of oligodendrocytes is lower than that of
358 astrocytes. Around 27.8% of oligodendrocytes were transduced by the AAV. We observed that
359 21.1% of transduced Olig2^{+} oligodendrocytes express TrkB.FL in the adult mouse brains (Fig. 9I-
360 M).

361 To find out whether oligodendrocytes also express TrkB.T, we quantified colocalization of
362 Olig2 and EGFP in tamoxifen-treated *Ntrk2*$^{CreER/+}$;GFPL10/+ mice. We found that 8.8% of Olig2^{+}
363 oligodendrocytes are colocalized with EGFP in the SS, indicating that these cells express either
364 TrkB.FL or TrkB.T in the adult mice. In the CA1 SR, 38.4% of Olig2^{+} oligodendrocytes express
365 either TrkB.FL or TrkB.T in the adult mice (Fig. 9R-U). Because we showed 21.1% of Olig2^{+}
366 oligodendrocytes express TrkB.FL in the CA1 SR of adult mice, this means that there are at least
367 another 17% of Olig2^{+} oligodendrocytes that express TrkB.T in the adult mouse brains. Olig2
368 labels both oligodendrocyte precursor cells (OPCs) and mature oligodendrocytes. To differentiate
369 between these two populations, we used aspartoacylase (ASPA) as a marker to mark mature
370 oligodendrocytes only. Our results show that nearly all mature oligodendrocytes do not express

371 either TrkB.FL or TrkB.T in the CA1 SR of adult mice, which suggests that only OPCs express

372 TrkB.FL and TrkB.T in adult mice (Fig. 9V-Y).



373

374 **Figure 9. OPCs express TrkB.FL and TrkB.T in adult mice.** (**A-G**) Immunostaining of Olig2 in the SS (A-C) and CA1

375 SR (E-G) of *Ntrk2*$^{2A\text{-}Cre/+}$;EGFP-L10a/+ mice, and (D,H) quantification of colocalization. (**I-M**) Immunostaining of Olig2

376 in the CA1 SR of adult *Ntrk2*$^{2A\text{-}Cre/+}$ mice injected with the mCherry-to-EGFP color switch AAV, and quantification of the

377 colocalization of GFAP and EGFP. (**N-U**) Immunostaining of Olig2 in the SS (A-C) and CA1 SR (E-G) of

378 *Ntrk2*$^{CreER/+}$;EGFP-L10a/+ mice, and (Q,U) quantification of colocalization. (**V-Y**) Immunostaining of ASPA in the CA1

379 SR (of *Ntrk2*$^{CreER/+}$;EGFP-L10a/+ mice, and (Y) quantification of colocalization. Scale bar = 50 µm. Arrowheads denote

380 EGFP-expressing oligodendrocytes. Arrows denote oligodendrocytes that do not express EGFP.

381

382 **4. Discussion**

383 Whether microglia express BDNF and TrkB or not has been debated. Microglia were
384 discovered to express BDNF *in vitro* at the level of mRNA [26, 27] and protein [28] decades ago.
385 LPS stimulation was suggested to increase BDNF secretion from cultured microglia [26, 28]. *In*
386 *vivo* experiments showed BDNF expression in activated microglia [29]. BDNF from spinal
387 microglia were suggested to be responsible for pain hypersensitivity [7]. Microglial BDNF was
388 also reported to promote motor learning-dependent synapse formation [6]. Following these
389 studies, many researchers showed the knockdown of microglial expression of BDNF disrupts
390 biological processes including self-renew and proliferation of hippocampal neurons [30], nerve
391 injury-induced pyramidal neuron hypersensitivity in the SS cortex [31], and mechanical allodynia
392 induced by high-frequency stimulation [32]. Microglial BDNF was also suggested to be critical for
393 (*R*)-ketamine-mediated microglial activation in the medial prefrontal cortex of chronic social defeat
394 stress mice [33]. However, other studies using $Bdnf^{Laz/+}$ reporter mice and transcriptomic analysis
395 show that microglia do not express significant level of BDNF in the spinal cord [8, 34]. This
396 inconsistency likely comes from the lack of binding specificity of BDNF antibodies, as many
397 previous efforts to characterize BDNF expression relied on immunohistochemistry. To avoid the
398 use of BDNF antibodies and increase sensitivity, we took advantage of the $Bdnf^{2A\text{-}cre/+}$ knockin
399 mice that express Cre recombinase under the *Bdnf* promoter and the EGFPL10a/+ reporter mice
400 that express EGFP localized in cell bodies but not dendrites and axons for better characterization
401 of colocalization. Our data suggest that both resting and activated microglia do not express BDNF
402 in the MO cortex, hippocampal CA1, and spinal cord. These results are consistent with findings
403 from transcriptomic analysis that showed microglia do not express BDNF in the spinal cord [8],
404 and from another group that recently demonstrated resting microglia and microglia activated by
405 ATP do not express BDNF transcriptionally and translationally in the mouse motor cortex [35].
406 Therefore, the phenotypes seen after deleting the microglial *Bdnf* gene likely come from artifacts.

407 However, our data do not exclude the possibility that microglia may have the potential to
408 internalize and recycle BDNF expressed by neurons and other cell types in the CNS.

409 Using similar method, we also show that microglia do not express the TrkB receptor in the
410 brain and spinal cord. Our data show microglia do not express TrkB.FL from embryo to adult, and
411 do not express TrkB.T in adult mice. This means that at least in adult mice, microglia do not
412 respond to BDNF through the TrkB receptor. However, it is still likely that BDNF can signal through
413 the low affinity receptor $p75^{NTR}$ in microglia, as studies have suggested a subset of microglia
414 express $p75^{NTR}$ and its expression is upregulated upon infection in mice [36]. Another study
415 suggested no significant $p75^{NTR}$ expression in wild-type microglia, but the expression is increased
416 in 5xFAD mice and Alzheimer's patients [37], suggesting a potential role of BDNF-$p75^{NTR}$ signaling
417 in neuroinflammation.

418 Cultured astrocytes have been shown to express BDNF and the expression is increased
419 by KCl, carbachol, and glutamate [38, 39]. Astrocytic BDNF regulates neuronal dendrite
420 complexity and spine number *in vitro* [16]. Studies have suggested that cortical layer II/III
421 astrocytes can incorporate extracellular proBDNF through $p75^{NTR}$-mediated endocytosis and
422 cleave proBDNF to mBDNF [15]. But whether and what percentage of astrocytes produce BDNF
423 *in vivo* under physiological condition remain elusive. Our results show that a small subset of
424 astrocytes express BDNF in the SS cortex (14.6%) and hippocampal CA1 SR (18.3%) under
425 physiological condition in mice. In adult mice, 10% of astrocytes in CA1 SR express BDNF. This
426 is consistent with other studies that showed a relatively low baseline level of mBDNF in astrocytes
427 [40], but an upregulation of astrocytic BDNF expression under pathological conditions induced
428 by MK-801, an N-methyl-D-aspartic acid (NMDA) receptor antagonist, *in vitro* [41] and treatment
429 of glatiramer acetate in R6/2 HD mouse model *in vivo* [40]. Although only 10% of astrocytes
430 express BDNF in the hippocampus under physiological condition, astrocytes seem to be an
431 important source of BDNF for normal CNS functions. Studies using BDNF knock-down mouse
432 models found that astrocytic BDNF is required for oligodendrogenesis after white matter damage

433 [42]. In the experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis,
434 astrocyte specific BDNF knockout led to more severe clinical course with increased axonal injury
435 and loss [43].

436 It is known that astrocytes predominantly express TrkB.T, which plays important roles in
437 mediating calcium release from intracellular stores in astrocytes [9] and controlling astrocyte
438 morphological maturation [11]. In the adult brain, only less than 5% astrocytes express TrkB.FL
439 in the hippocampal CA1 SR. These data are consistent with the current belief that astrocytes
440 dominantly express TrkB.T in the adult mice. We also show that cumulatively through embryo to
441 adult, 18% astrocytes in the hippocampal CA1 SR have expressed TrkB.FL, suggesting it is likely
442 that some astrocytes only express TrkB.FL early during embryonic and postnatal developmental
443 stages. This corroborates a previous *in vitro* study that showed radial glia (the main astrocytic
444 precursor cells) and proliferating cortical astrocytes in culture express mRNAs for both TrkB.FL
445 and TrkB.T, whereas differentiating astrocytes predominantly express TrkB.T and downregulate
446 TrkB.FL expression [44]. These data suggest that BDNF-TrkB.FL signaling may play a role in
447 astrocyte development and maturation.

448 Our data show that a very small subset (less than 5%) of oligodendrocytes in the
449 hippocampal CA1 SR express BDNF. Contradictory to the studies that showed BDNF expression
450 in cortical oligodendrocytes [45, 46], our data showed that most Olig2^{+} cells in the cortex do not
451 express BDNF. Oligodendroglial BDNF expression has been reported by a few studies. A study
452 has showed that oligodendrocytes in the spinal cord and optic nerve express BDNF mRNA and
453 the expression is increased in Long-Evans shaker rats that lack CNS myelin due to the MBP gene
454 mutation [47]. Another study found that fingolimod treatment resulted in increased BDNF mRNA
455 concomitant with increased OPC differentiation in rat OPC culture [48]. Our results confirm the
456 expression of BDNF in oligodendrocytes in certain brain regions. And our observation of
457 increased BDNF-expressing oligodendrocytes in AAV injected mice may suggest that BDNF

458 expression is increased when myelin is disrupted, as a previous study has reported increased
459 BDNF level in oligodendrocytes after injury [49].

460 Our results also showed that only OPCs, but not mature oligodendrocytes, express
461 TrkB.FL and TrkB.T in the hippocampal CA1 SR of adult mice. BDNF-TrkB signaling in OPCs has
462 been suggested to have important functions. OPC-specific TrkB deletion resulted in impaired
463 activity-dependent myelination [18]. BDNF and its structural mimetic TDP6 have been shown to
464 promote myelination and myelin repair via oligodendroglial TrkB receptor [20, 50, 51]. Future
465 studies are necessary to determine which TrkB isoform(s) are responsible for mediating these
466 effects.

467 In summary, we characterized the expression of BDNF and TrkB in microglia, astrocytes,
468 and oligodendrocytes in the current study. Our results clarify that microglia in the examined
469 regions do not express BDNF or TrkB. Our findings also open opportunities to investigate
470 previously unidentified roles of BDNF and TrkB in astrocytes and oligodendrocytes.

471

472

473 **Author Contributions**

474 C.N. and B.X. designed research; C.N., X.Y., J.J.A., and H.X. performed experiments; C.N.
475 analyzed data; C.N. wrote and B.X. edited the paper.

476

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480

481 **Data Availability Statement**

482 Original data generated and analyzed during this study are included in this published article.

483

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487

488 **Conflicts of Interest**

489 The authors declared that they have no competing financial interests.

490

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