Murine Olfactory Bulb Interneurons Survive Infection with a Neurotropic Coronavirus

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Abstract. Viral infection of the central nervous system is complicated by the mostly irreplaceable nature of neurons, as the loss of neurons has the potential to result in permanent damage to brain function. However, whether neurons or other cells in the CNS sometimes survive infection and the effects of infection on neuronal function are largely unknown. To address this question, we used the rJHM strain (rJ) of mouse hepatitis virus, (MHV), a neurotropic coronavirus, which causes acute encephalitis in susceptible strains of mice. To determine whether neurons or other CNS cells survive acute infection with this virulent virus, we developed a recombinant JHMV that expresses Cre recombinase (rJ-Cre) and infected mice that universally expressed a silent (floxed) version of tdTomato. Infection of these mice with rJ-Cre resulted in expression of tdTomato in host cells. The results showed that some cells were able to survive the infection, as demonstrated by continued tdTomato expression after virus antigen could no longer be detected. Most notably, interneurons in the olfactory bulb, which are known to be inhibitory, represented a large fraction of the surviving cells. In conclusion, our results indicated that some neurons are resistant to virus-mediated cell death and provide a framework for studying the effects of prior coronavirus infection on neuron function.

Importance. We developed a novel recombinant virus that allows for the study of cells that survive an infection by a central nervous system-specific strain of murine coronavirus. Using this virus, we identified neurons and to a lesser extent, non-neuronal cells in the brain that were infected during the acute phase of the infection and survived for approximately two weeks until the mice succumbed to the infection. We focused on neurons and glial cells within the olfactory bulb because the virus enters the brain at this site. Our results show that interneurons of the olfactory bulb were the primary cell type able to survive infection. Further, these results indicate

41 that this system will be useful for functional and gene expression studies of cells in the brain that
42 survive acute infection.

INTRODUCTION

Viral upper respiratory infection is a common cause of olfactory dysfunction, in part because the olfactory epithelium is located adjacent to respiratory epithelium, the site of replication of multiple viruses that cause upper respiratory tract infection and because olfactory neurons directly access the environment. Viruses take advantage of this direct connection with the olfactory bulb (OB) to enter the central nervous system (CNS) (1-6). In the process of gaining access to the CNS, these viruses damage the olfactory epithelium and the olfactory bulb leading to altered olfaction (7-11).

The process of scent discrimination begins within the olfactory epithelium when odorants bind odorant receptors on olfactory sensory neurons (OSNs) (12, 13). OSNs project their axons onto the dendrites of projection neurons (tufted cells and mitral cells) within the olfactory glomeruli of the OB. These tufted and mitral cells then send axons deeper into the brain, largely to the primary olfactory cortex but also to secondary and tertiary connections of the OB. Interneurons within all the layers of the olfactory bulb modulate the signal sent by these projection neurons. While all olfactory bulb interneurons use gamma-aminobutyric acid as a neurotransmitter, some also express dopamine. These interneurons, which include granule cells and periglomerular cells, are characterized by soma size, soma location, dendrite extension, and expression of calcium-binding proteins (14-17). For example, periglomerular cells have a small soma, are located in the glomerular layer of the olfactory bulb and express tyrosine hydroxylase, calbindin, or calretinin. In contrast, granule cells express calretinin but not calbindin or tyrosine hydroxylase (14, 18, 19). While these interneurons are inhibitory by nature, and anatomical studies have shown that each subtype extends dendrites, little is known about the function of these cells or how they are molecularly distinct from each other.

66 Coronaviruses (CoV) are positive-stranded RNA viruses capable of causing disease in a
67 variety of animals. These diseases range from respiratory, systemic, neurological and
68 gastrointestinal diseases in domestic, companion and experimental animals to mild and severe
69 respiratory disease in humans (20, 21). Neurotropic strains of the murine CoV, mouse hepatitis
70 virus (MHV), cause acute encephalitis and acute and chronic demyelinating diseases of the
71 central nervous system (22). In specific, the non-recombinant and recombinant (rJ) versions of
72 neurotropic JHM cause lethal encephalitis. When this virus is intranasally instilled, virus enters
73 the CNS through the OB by direct infection of OSNs and anterograde transport via the olfactory
74 nerve. Once in the OB, JHMV spreads trans-neuronally to connections of the main OB (23, 24).
75 Unlike other neurotropic strains of MHV, JHMV primarily infects neurons (25-28). However,
76 little is known about the ratio of neuronal to glial infection compared or about the subtypes of
77 neurons infected by JHMV, although infection of tyrosine hydroxylase-expressing neurons may
78 be limited to certain regions of the brain (24).

79 The irreplaceable nature of most neurons is a major factor in the long-term morbidity
80 observed after viral infections of the central nervous system. Loss of individual neurons and the
81 associated disruption of interconnected neural networks results in permanent damage to the
82 brain. Though not extensively validated, it would be advantageous for neurons to survive after
83 viral infection. In support of this, neurons have been shown to survive an attenuated rabies virus
84 infection (29) but whether this phenomenon occurs with viruses other than attenuated rabies
85 virus is not known.

86 Here, to study brain cells that survive infection after neurotropic CoV infection, we
87 developed a recombinant JHM virus that expressed Cre recombinase (Cre). tdTomato mice
88 contain a transgenic tdTomato cassette in a locus that is universally expressed (*Rosa26* locus)

thus allowing for expression of the fluorescent protein tdTomato after Cre-LoxP-mediated excision of a stop cassette (30). Viral Cre expression within the infected host cell results in excision of a stop cassette, leading to expression of the red fluorescent protein tdTomato only in infected cells. Because the host cell contains the tdTomato cassette, cells surviving the infection permanently express tdTomato even after virus is eliminated. Using this model to assess survival of neurons after virus clearance, we identified a population of OB interneurons that survive the infection.

RESULTS

Construction of a Cre-expressing recombinant JHMV (rJ-Cre). Although it is established that rJ infects neurons, including mitral cells (25, 26), it is not known whether any neurons in general survive this infection, or whether certain neuronal cell types preferentially survive. To engineer a Cre-expressing rJ virus, we used a previously described system of reverse genetics utilizing a Bac cDNA clone (pBAC-JHMV^{IA}) (31). Cre was inserted into pBAC-JHMV^{IA} in place of ORF4, a gene that is dispensable for viral replication in tissue culture or in mice (32, 33) using Red recombination with an arabinose-inducible *Flp* recombinase (**Figure 1A**). rJ-Cre was propagated and analyzed for its ability to replicate in tissue culture cells and to cause lethal encephalitis in mice. Insertion of the *Cre* gene had little to no effect on virus replication in 17Cl-1 cells compared to wild-type rJ (**Figure 1B**). Infection of C57Bl/6 mice with 4 x10^4 rJ-Cre resulted in morbidity and mortality indistinguishable from that seen in mice infected with rJ (**Figure 1C**). Together, these results indicate that the insertion of Cre into the rJ genome did not appreciably alter viral fitness.

To assess the functionality of Cre expressed from rJ-Cre, tdTomato mice were intranasally infected with 4 x10^4 rJ-Cre. After intranasal infection, rJ accesses the brain by replication in the

olfactory receptor neurons and anterograde travel to the neurons of the olfactory bulb. Virus then spreads transneuronally throughout the brain via primary, secondary and tertiary connections of the OB, reaching sites in the brainstem, amygdala and midbrain by 7 days post-infection (dpi) (23, 24). In preliminary experiments, we observed that approximately seven days were required after inoculation of the animal before tdTomato expression was sufficiently elevated to be detected by confocal microscopy. By 11 dpi, robust tdTomato levels could be detected by confocal microscopy in neurons of the olfactory system, including neurons in the brainstem at sites known to be tertiary connections of the OB (**Figure 2A**). Periglomerular cells (arrows, right panel) were often dege nerate (i.e. nuclei were small and hyperchromatic). These changes were seen on a background of moderate cellular inflammation (**Figure 2B**). These results indicated that, as expected, rJ-Cre expressed Cre recombinase *in vivo* and expression levels of tdTomato were sufficient for studying cells that survived the acute infection.

tdTomato-expressing cells remain after virus clearance. To determine more precisely the temporal relationship between virus infection and tdTomato positivity, we harvested brains at 4, 7, and 11 dpi and assessed tdTomato and viral nucleocapsid (N) protein expression using confocal microscopy. In preliminary studies, we noted that tdTomato expression lagged behind that of viral antigen, likely reflecting the requirement for Cre expression, transport to the nucleus, DNA excision and mRNA translation before protein can be expressed. Consequently, we focused our studies on the OB because this is the first site of virus replication. Further, JHMV and other strains of MHV show a preference for replicating in the OB even when virus is introduced intracranially, as virus titers are often highest in this part of the brain (10, 11, 34). After staining OB sections with anti-N MAb at 4 dpi, neither viral antigen nor tdTomato was detected in the olfactory bulb (**Figure 3A**). However, distinct tdTomato^{+} and

N protein$^+$ cells were apparent by 7 dpi. N protein was detected in spite of the increase in auto-fluorescence seen upon virus infection. At 7 dpi, most tdTomato colocalized with N protein though some strongly tdTomato$^+$, N protein negative cells were clearly visible (**Figure 3B**). However, by 11 dpi, viral antigen was no longer detected within the OB even as tdTomato expression became more prominent (**Figure 3A**).

As additional support for the notion that the presence of tdTomato$^+$ cells reflected cell survival after virus clearance, we measured levels of viral RNA in the OB. Viral subgenomic RNA within the olfactory bulb was detected at 3 dpi, prior to the detection of virus antigen or tdTomato positivity and reached peak levels at 5 dpi (**Figure 3C)**. Levels of subgenomic RNA then declined and were detected at low levels at 7 dpi indicating that viral clearance was occurring. The presence of tdTomato-expressing cells in the OB even as virus was cleared from this site supports the conclusion that at least a subset of CNS cells was able to clear the infection and remain viable.

Interneurons of the olfactory bulb survive rJ infection. As expected, given the cellular tropism of rJ, most surviving tdTomato-positive cells were neurons as demonstrated morphologically and confirmed by NeuroTrace staining (**Figure 4**). Some neurons within the olfactory bulb strongly expressed tdTomato throughout the cell. As demonstrated by morphology, neurons surviving rJ-Cre infection were largely interneurons (**Figure 4B**); no mitral cells were tdTomato-positive perhaps indicating that mitral cells did not survive rJ infection. Surviving interneurons were primarily located in the glomerular cell layer and granule cell layer of the olfactory bulb (**Figure 5A**). These results indicate that interneurons comprised a large fraction of cells that survived rJ infection, suggesting an increased ability to survive the viral infection.

158 Interneurons of the olfactory bulb are classified based on their location and expression of
159 neurotransmitters and other cell markers. For example, periglomerular interneurons of the
160 glomerular cell layer can be calretinin, tyrosine hydroxylase or calbindin-positive. To define
161 more precisely the type of interneuron surviving rJ-Cre infection, we stained olfactory bulbs with
162 antibodies to tyrosine hydroxylase, calretinin, and parvalbumin. tdTomato-positive cells did not
163 express any of these markers. While readily detected, tyrosine hydroxylase-expressing cells did
164 not co-express tdTomato (**Figure 5B**). Similarly, tdTomato$^+$ parvalbumin$^+$ and tdTomato$^+$
165 calretinin$^+$ cells also were not detected (**Figure 5C, D**). These results suggest that expression of
166 the cell-specific marker was decreased in surviving cells, or alternatively, infection was
167 predominantly of an interneuron subset not expressing one of the three proteins that we assayed.

168 **Rare glial cells survive rJ infection.** rJ is known to primarily infect neurons (25, 26) and
169 tdTomato$^+$ cells were morphologically neurons; however, rJ infection of glia has also been
170 reported (28, 35, 36). To better characterize the relative proportion of neuronal and nonneuronal
171 cells in the brain that survive infection, we stained sections from infected tdTomato$^+$ mice with
172 antibodies specific for astrocytes and microglia. First, sections were stained with antibody to
173 glial fibrillary acidic protein (GFAP), a well-described protein expressed by astrocytes. The
174 results showed that few astrocytes were tdTomato$^+$ although, a few tdTomato$^+$ astrocytes were
175 found (**Figure 6A**). To determine whether microglia survived rJ-Cre infection, we
176 immunostained OB sections with an antibody to IBA-1, a protein that is upregulated on these
177 cells at sites of inflammation. Some cells exhibited co-localization of IBA-1 and tdTomato after
178 infection (**Figure 6B**), but the pattern of co-localization appeared punctate and phenotypically
179 different from the diffuse tdTomato expression detected in neurons. Therefore, this punctate
180 pattern may represent microglia/macrophage phagocytosis of tdTomato$^+$ cells as opposed to *de*

novo tdTomato expression. Consistent with this interpretation, there was a lack of tdTomato positivity detected in the nucleus of these cells. To confirm these results, we bred CX3CR1-GFP, which serve as microglia-reporter mice, to tdTomato mice. F1 progeny from this cross were infected with rJ-Cre. These mice constitutively express GFP in microglia/macrophages, and will express tdTomato in cells after viral infection, eliminating the need for immunostaining. Experiments with CX3CR1$^{GFP/+}$ tdTomato$^{+/-}$ mice largely recapitulated the punctate IBA1 immunostaining described above. Microglia/macrophages with a punctate pattern of tdTomato were near tdTomato^{+} neurons (**Figure 6C)**. However, uncommon cells with the typical morphology of microglia showing a more diffuse pattern of tdTomato expression in both the cytoplasm and nucleus were also found, suggestive of rare endogenous infection (**Figure 6C**). Collectively these results indicate that rJ is capable of infecting glial cells, albeit at low levels, and that some astrocytes and microglia survive rJ infection. They also suggest, perhaps not surprisingly, that microglia/macrophages play a role in clearing virus-infected cells.

DISCUSSION

While the consequences of viral infection in the central nervous system can be devastating because of neuronal loss, little is known about whether neurons that survive are dysfunctional. The main challenge to studying neurons affected by virus infection is to identify and isolate those cells after virus has been cleared. Here, we demonstrate a method useful for identifying previously infected cells by alteration of the host genome using virally-expressed Cre protein. We used a virulent neurotropic CoV that results in a lethal disease by 12 dpi. We found cells, especially in the OB, that survived the infection at times when viral antigen could no longer be detected by immunostaining. These results corroborate previous studies in which populations of cells that survive infection were identified using Cre-based methodology. In one such study,

small numbers of cells, found to be club cells by mRNA sequencing, survived influenza A virus infection and had increased levels of interferon-stimulated proteins, resulting in an inflammatory disease (37). In another study, an attenuated Rabies virus-expressing-Cre reporter system showed that neurons survived up to 6 months after infection. Surviving neurons in this study showed alterations in transcripts for neuronal function and structure by microarray analysis (29). Our results using a virulent strain of JHMV indicate that neurons can even survive infection with a highly pathogenic virus.

Our results provide proof of principle for using a Cre-reporter model for studying cells surviving MHV infection in the central nervous system. Additionally, GFAP and IBA1 staining confirmed that neurons preferentially survive rJ infection since few astrocytes or microglia expressed tdTomato after infection. These results are consistent with previous work demonstrating a tropism for neuronal cells, but it is also possible that glial cells survive infection less frequently. Future work based on the method described herein will be useful for studying the CNS of mice infected with CoV with different cellular tropisms. Of particular interest will be the consequences of infection with the neuroattenuated J2.2-V-1 strain of JHMV, which preferentially infects oligodendrocytes and causes clinically apparent demyelinating disease. A Cre-expressing recombinant J2.2 would allow for study of the effects of viral infection on oligodendrocyte RNA and protein expression and would provide new information on the effects of prior infection on demyelination and remyelination. Most studies have focused on gross areas of myelin destruction but such a recombinant virus would facilitate analyses of surviving and possibly dysfunctional cells.

Many viruses replicate in the nasal cavity and the olfactory epithelium, which is distinct from the respiratory epithelium, and serves as an important portal of virus entry into the CNS (1).

Thus, virus is first detected in the OB in experimental infections caused by neurotropic influenza A virus, West Nile virus and others (2-6). In a similar vein, the OBLV strain of MHV replicates to high titers in the OB, with little evidence of spread elsewhere in the brains of immunocompetent mice (10, 11, 34). However, in mice lacking T or B cells OBLV spreads throughout the brain (34). The propensity for viruses to invade the CNS via the OB, combined with the uncommonness of viral encephalitis, suggests that the olfactory epithelium, nerve, or bulb may limit viral spread to and within the CNS, perhaps by modulating the immune response. Our results indicate that some cells in the OB, especially interneurons, survive the initial virus infection. These cells are primarily inhibitory and modulate neuronal function. Whether these surviving neurons have diminished function, resulting in changes in olfaction will require additional investigation.

MATERIALS AND METHODS

Cell culture. MHV-receptor-expressing HeLa cells (HeLa-MHVR), 17Cl-1 cells, and MHV-receptor expressing BHK cells were grown as previously described (38, 39).

Generation of recombinant JHMV-Cre. Cre recombinase was cloned into pBAC-JHMV as previously described (31). Briefly, a PCR product containing Cre-FRT-Kan^{r}-FRT and 5' and 3' homology to the regions just outside ORF4 was created using two-step PCR. A plasmid containing Cre sequence was a gift from Benjamin tenOever (Icahn School of Medicine). This cassette was transformed into *E. coli* containing pBAC-JHMV^{IA}. Bacteria with successfully recombined pBAC-JHMV were identified by kanamycin resistance. Correct clones were amplified and treated with *Flp* recombinase to excise the kanamycin resistance cassette surrounded by Flp recombination targets. pBAC-derived JHMV-Cre was obtained after transfection as previously described (31). rJ-Cre virus was grown on 17Cl-1 cells, and virus titers

were determined on HeLa-MHVR cells (40). 17Cl-1 cells were infected with rJ at a multiplicity of infection (MOI) of 0.1, and virus from the supernatant and cells was combined prior to determining viral titers. Virus was passaged five times to obtain sufficient stocks to use in mouse experiments and an additional three times to assess stability of the Cre insertion. Levels of Cre expression were unchanged through 7 passages but were diminished by passage 8, indicating some instability of the Cre gene.

Mice. Specific-pathogen-free C57Bl/6 mice were purchased from Charles River. B6.Cg-*Gt(ROSA)26Sor*$^{tm14(CAG-tdTomato)Hze}$/J (tdTomato) mice were purchased from Jackson Laboratories. B6.129P(Cg)-*Ptprc*^{a} *Cx3cr1*^{tm1Litt}/LittJ (CX3CR1-GFP) mice were also purchased from Jackson Laboratories and bred to tdTomato mice. Mice were maintained in specific-pathogen-free facilities at The University of Iowa. Male mice were used in all experiments. 5-6-week-old mice were intranasally inoculated with 40,000 PFU rJHM-Cre after isofluorane anesthesia. After viral inoculation, mice were observed and weighed daily. To titer virus from infected animals, mice were sacrificed and perfused with phosphate-buffered saline (PBS). Brain tissue was homogenized into PBS using a manual homogenizer and frozen. After thawing, cellular debris was removed by centrifugation, and virus titers in the supernatant were determined on HeLa-MHVR cells. The University of Iowa Institutional Animal Care and Use Committee approved all mouse experiments.

RNA analysis. Olfactory bulbs were collected at indicated times and placed into Trizol (Thermo Fisher Scientific). RNA was isolated according to the manufacturer's instructions. RNA was transcribed into cDNA using Moloney murine leukemia virus reverse transcriptase (MMLV RT) (Thermo Fischer Scientific). Subgenomic RNA levels were measured on a QuantStudio qPCR 3 system (Thermo Fisher Scientific) using previously described subgenomic RNA primers (41).

The levels of subgenomic RNA were normalized to hypoxanthine-guanine phosphoribosyltransferase (HPRT) by the following threshold cycle (C_T) equation: $\Delta C_T = C_T$ of gene of interest − C_T of HPRT. All results are shown as a ratio to HPRT calculated as $2^{-\Delta CT}$.

Tissue processing. After perfusion of the mouse, brains were transferred to 4% paraformaldehyde solution in a 20:1 volume to weight ratio. After 48 hours, brains were cryoprotected by immersion in 10% sucrose for 30 minutes, followed by immersion in 20% sucrose for several hours until brains had dropped to the bottom of the solution. Then, brains were transferred to 30% sucrose and kept at 4°C overnight. Brains were snap-frozen in tissue freezing media using a stand-alone Gentle Jane device. 10 or 50 µM sections were obtained on a Thermo cryostat and stored at -80°C. For hematoxylin and eosin staining, brains were removed, fixed in zinc formalin, then embedded in paraffin. Tissue sections were stained with H&E.

Tissue staining and imaging. For staining, frozen sections were warmed at room temperature for 10 minutes. Sections were immersed in PBS for 10 minutes before a 10-minute treatment with 0.1% Triton-X in PBS. Sections were then rinsed in PBS 3x for 5 minutes each. Next, samples were incubated in CAS block (Invitrogen) for 10 minutes followed by incubation in primary antibody diluted in 1% goat serum in PBS overnight at 4°C in a humidity chamber. Primary antibodies to GFAP (Sigma) at 1:10000, IBA1 (Wako) at 1:2000, parvalbumin (Sigma) at 1:1000, tyrosine hydroxylase (Millipore) at 1:1000, calretinin (Millipore) at 1:1000 and viral N protein (kindly provided by Dr. Michael Buchmeier, University of California, Irvine) at 1:10000 were used. Sections were rinsed before incubation with a 1:200 dilution of an appropriate A488-conjugated goat anti-mouse or anti-rabbit antibody, Thermo Fisher Scientific). In some cases, Topro-3 (Thermo Fisher Scientific) was included in the secondary antibody staining solution at a 1:1000 dilution. After rinsing with PBS, slides were mounted with

Vectashield anti-fade reagent (Vectashield Laboratories); in some experiments Vectashield containing DAPI was used. NeuroTrace (Thermo Fisher Scientific) staining was performed following the manufacturer's protocol. Images were obtained using a Zeiss LSM510 confocal microscope or an Olympus BX61 light microscope.

Statistics. Data are presented as mean ± SEM unless otherwise indicated. Mann-Whitney *U* tests were used to analyze differences in means. Log-rank tests were used to determine significant differences in survival of mice. $p < 0.05$ were considered significant.

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FIGURE LEGENDS

Figure 1. Characterization of Cre-expressing rJ. A) Genome of rJ and recombinant rJ expressing Cre recombinase. B) Replication kinetics of rJ-Cre and rJ. 17Cl-1 cells were infected at an MOI of 0.1 PFU/cell. Virus titers were determined as described in Materials and Methods. C) Mice were intranasally infected with 4 x 10^4 PFU of the indicated virus and monitored daily for survival. Data shown are from one experiment representative of two independent experiments with 5 mice/group.

Figure 2. Visualization of tdTomato-positive cells and brain pathology in brains after rJ.Cre infection. A) Eleven dpi, brains from Cre-reporter tdTomato mice were harvested, cryosectioned and imaged without any additional staining. Images are representative of analyses of 7 mice. B) Histology of olfactory bulbs in naïve and rJ-Cre infected animals at 11 dpi. White arrow in the inset on the left indicates a healthy periglomerular cell; black arrow in right-sided inset indicates nuclear changes seen in periglomerular cells. All images are shown at 10X magnification; insets were cropped to show an individual glomerulus.

Figure 3. Temporal detection of tdTomato and N protein-positive cells. A) Brains from naïve and rJ-Cre-infected tdTomato mice were harvested, cryosectioned, and visualized after staining with a monoclonal antibody to the viral N protein at 4, 7 and 11 dpi. All images are from the olfactory bulb region. B) Enlargement of boxed area shown in panel A (red arrow indicates tdTomato$^+$N$^-$ cell while white arrow shows tdTomato$^-$N$^+$ cell; the remainder of the cells are

positive for both tdTomato and N protein. C) RNA was isolated from the olfactory bulbs of infected mice at the indicated time points. A quantitative PCR assay was used to determine levels of sub-genomic viral RNA. Expression of sub-genomic RNA was normalized to HPRT. Data shown represent 4-5 mice at each timepoint.

Figure 4. tdTomato-positive cells are largely neurons. A) rJ-Cre-infected tdTomato mice were euthanized at 11 dpi. Cryosections from the indicated areas of the brain were stained with the fluorescent Nissl stain NeuroTrace. B) High-power images of olfactory bulb interneurons stained with NeuroTrace. Images are representative of 3-5 mice.

Figure 5. Surviving tdTomato-positive cells in the OB are are primarily interneurons. rJ-Cre-infected brains were harvested, cryosectioned and stained as indicated. A) Low-power view of the olfactory bulb showing the anatomical location of surviving tdTomato-positive cells. The glomerular layer (GL) and granule cell layer (GCL) are labeled. B-D) Tyrosine hydroxylase (B), Parvalbumin (C), and Calretinin (D) antibody staining of cryosectioned olfactory bulbs. All images are from the olfactory bulb region at 11 dpi and are representative of 3-5 mice.

Figure 6. A small fraction of glia is tdTomato-positive. Brains from rJ-Cre infected tdTomato mice were harvested at 11 dpi, cryosectioned and stained with antibodies to detect astrocytes (A, GFAP) or microglia (IBA1, B). C) Brains from tdTomato$^{+/-}$ CX3CR1$^{GFP/+}$ mice were harvested at 11 dpi, cryosectioned and visualized after Topro-3 nuclear staining. White arrows indicate surviving, double-labeled cells (A, B, and C). Yellow arrows in panel C indicate punctate tdTomato, CX3CR1-GFP double-labeling.