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Supplemental Information

The Chemokine Receptors Ccr5 and Cxcr6 Enhance Migration of Mesenchymal Stem Cells into the Degenerating Retina

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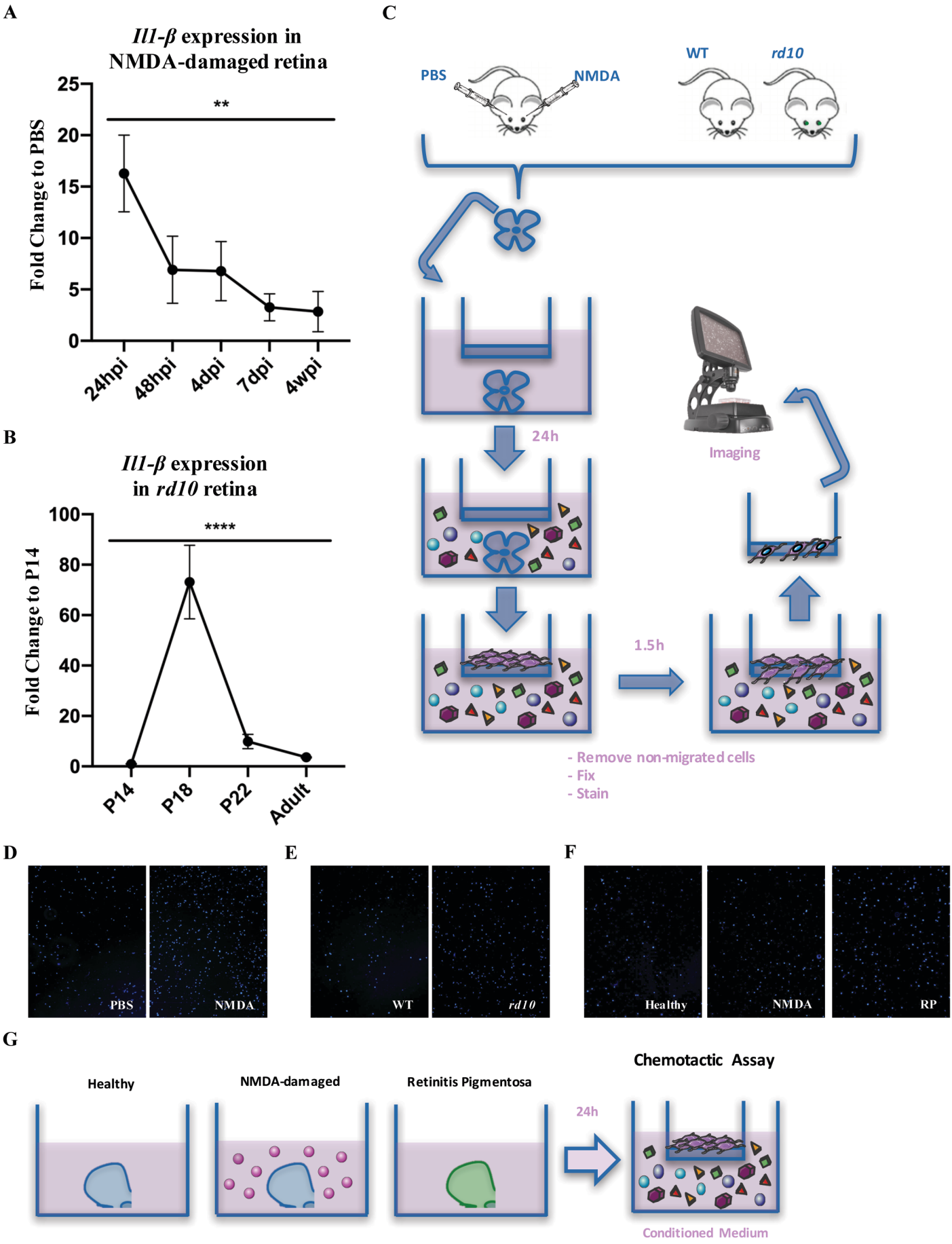
A
Il1-β expression in NMDA-damaged retina
Fold Change to PBS
25
20
15
10
5
0
**
24hpi
48hpi
4dpi
7dpi
4wpi
B
Il1-β expression in rd10 retina
Fold Change to P14
100
80
60
40
20
0
****
P14
P18
P22
Adult
C
PBS
NMDA
WT
rd10
24h
Imaging
1.5h
- Remove non-migrated cells
- Fix
- Stain
D
PBS
NMDA
E
WT
rd10
F
Healthy
NMDA
RP
G
Healthy
NMDA-damaged
Retinitis Pigmentosa
24h
Chemotactic Assay
Conditioned Medium

Fig. S1

Fig. S1. Retinal damage is associated with an acute peak in *Il1-β* expression. qRT-PCR of *Il1-β* levels in the **(A)** NMDA-damaged retina (24 hpi, 48 hpi, 4 dpi, 7 dpi, 4 wpi) and in the **(B)** *rd10* retina (P14, P18, P22, adult). Transcript levels are expressed as fold-changes to control (PBS-injected or P14) retinae. Data are presented as mean ± SEM from $n \geq 3$ independent experiments. One Way Anova was used for statistical analysis. **(C)** Experimental scheme of chemotactic assays with murine samples. WT mice received intravitreal injection of NMDA to induce retinal degeneration; the contralateral eye was injected with PBS, as a control. PBS/NMDA-injected animals were sacrificed 24 hpi. *Rd10* mice (and their age-matched WT controls) were instead sacrificed at P18. Eyecups were then cultured for 24h in SF medium. At the moment of the assay, a suspension of $2*10^5$ MSC in SF medium was seeded in the upper chamber. Following incubation, non-migrated cells were removed. Migrated cells stuck in the porous membrane of the transwell were stained with DAPI, imaged and quantified. Representative DAPI-stained fields from PBS vs NMDA **(D)**, and from WT vs *rd10* **(E)** transwell assays. **(F)** Representative DAPI-stained fields from healthy, NMDA and RP transwell assays performed with human samples. **(G)** Experimental scheme of chemotactic assays with human samples. The central part of the retina was divided into quarters. For each of the experiments, a quarter was cultured in control medium, while another one was cultured in medium containing NMDA (1mM). Similarly, a quarter from a healthy control and a quarter from a RP retina were cultured in parallel. After 24h, the conditioned medium was collected and used to perform chemotactic assays.

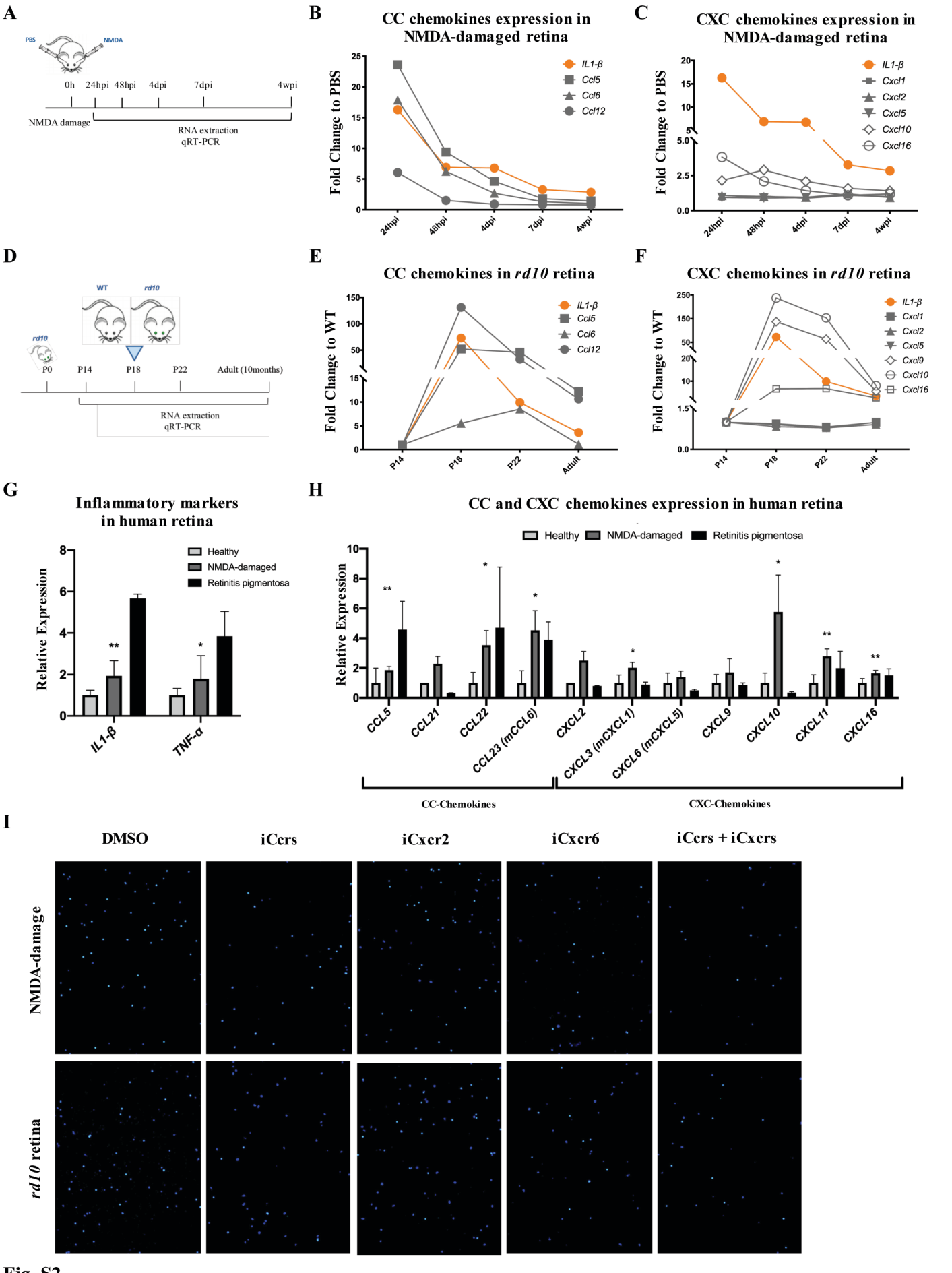
A
PBS
NMDA
0h
24hpi
48hpi
4dpi
7dpi
4wpi
NMDA damage
RNA extraction
qRT-PCR
B
CC chemokines expression in NMDA-damaged retina
Fold Change to PBS
IL1-β
Ccl5
Ccl6
Ccl12
24hpi
48hpi
4dpi
7dpi
4wpi
C
CXC chemokines expression in NMDA-damaged retina
Fold Change to PBS
IL1-β
Cxcl1
Cxcl2
Cxcl5
Cxcl10
Cxcl16
24hpi
48hpi
4dpi
7dpi
4wpi
D
WT
rd10
rd10
P0
P14
P18
P22
Adult (10months)
RNA extraction
qRT-PCR
E
CC chemokines in rd10 retina
Fold Change to WT
IL1-β
Ccl5
Ccl6
Ccl12
P14
P18
P22
Adult
F
CXC chemokines in rd10 retina
Fold Change to WT
IL1-β
Cxcl1
Cxcl2
Cxcl5
Cxcl9
Cxcl10
Cxcl16
P14
P18
P22
Adult
G
Inflammatory markers in human retina
Relative Expression
Healthy
NMDA-damaged
Retinitis pigmentosa
IL1-β
TNF-α
H
CC and CXC chemokines expression in human retina
Healthy
NMDA-damaged
Retinitis pigmentosa
Relative Expression
CCL5
CCL21
CCL22
CCL23 (mCCL6)
CXCL2
CXCL3 (mCXCL1)
CXCL6 (mCXCL5)
CXCL9
CXCL10
CXCL11
CXCL16
CC-Chemokines
CXC-Chemokines
I
DMSO
iCcrs
iCxcr2
iCxcr6
iCcrs + iCxcrs
NMDA-damage
rd10 retina

Fig. S2

Fig. S2. Retina degeneration is associated with time-dependent inflammation and increased expression of specific CC and CXC inflammatory chemokines. (A) Experimental scheme indicating analyzed time-points for PBS- and NMDA-injected (24 hpi, 48 hpi, 4 dpi, 7 dpi, 4 wpi). Time course analysis of *Il1-β* and either of *CC* **(B)** or *CXC* **(C)** chemokines expression in NMDA-damaged retinae. Transcript levels are expressed as fold-change to control (PBS-injected) retinae. Data are presented as mean from n ≥ 3 independent experiments. **(D)** Experimental scheme indicating analyzed time-points for WT and *rd10* retinae (P14, P18, P22, adult). Time course analysis of *Il1-β* and either of *CC* **(E)** or *CXC* **(F)** chemokines expression in *rd10* retinae. Transcript levels are expressed as fold-change to P14 retinae. Data are presented as mean from n ≥ 3 independent experiments. **(G)** qRT-PCR of pro-inflammatory cytokines *IL1-β* and *TNF-α* in healthy, NMDA-damaged and RP human retinae, cultured for 24h. Transcript levels are expressed as fold-change to the healthy controls. Data are presented as mean ± SD from n ≥ 3 (NMDA-damaged) or n = 2 (RP) independent experiments. Mann-Whitney test was used for statistical analysis. **(H)** qRT-PCR of *CC* and *CXC* chemokines levels in healthy, NMDA-damaged and RP human retinae, cultured for 24h. Transcript levels are expressed as fold-change to the healthy controls. Data are presented as mean ± SD from n ≥ 3 (NMDA-damage) or n = 2 (RP) independent experiments. Mann-Whitney test was used for statistical analysis. **(I)** Representative DAPI-stained fields from transwell assays assessing migration of mMSCs towards either NMDA-damaged (top) or *rd10* (bottom) retinae, in the presence of DMSO (vehicle), iCxcr2, iCcrs (Ccr1, Ccr3, Ccr5), iCxcr6 or iCcrs (Ccr1, Ccr3, Ccr5) + iCxcrs (iCxcr2 + iCxcr6).

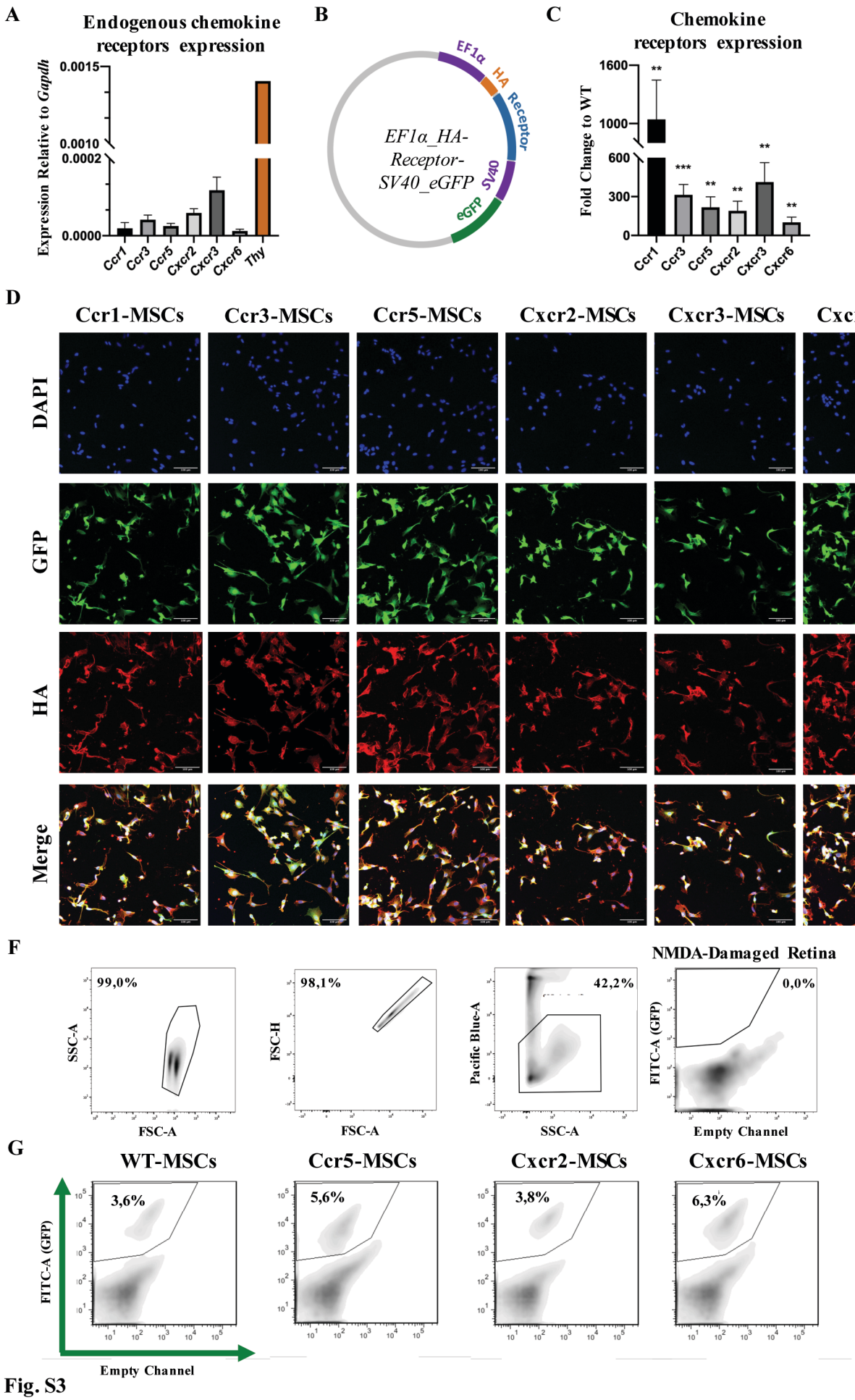
A
Endogenous chemokine receptors expression
Expression Relative to Gapdh
0.0015
0.0010
0.0002
0.0000
Ccr1
Ccr3
Ccr5
Cxcr2
Cxcr3
Cxcr6
Thy
B
EF1α
HA
Receptor
SV40
eGFP
EF1α_HA-Receptor-SV40_eGFP
C
Chemokine receptors expression
Fold Change to WT
1600
1000
600
300
0
**
***
**
**
**
**
Ccr1
Ccr3
Ccr5
Cxcr2
Cxcr3
Cxcr6
D
Ccr1-MSCs
Ccr3-MSCs
Ccr5-MSCs
Cxcr2-MSCs
Cxcr3-MSCs
Cxcr6-MSCs
DAPI
GFP
HA
Merge
F
NMDA-Damaged Retina
99,0%
SSC-A
FSC-A
98,1%
FSC-H
FSC-A
42,2%
Pacific Blue-A
SSC-A
0,0%
FITC-A (GFP)
Empty Channel
G
WT-MSCs
Ccr5-MSCs
Cxcr2-MSCs
Cxcr6-MSCs
3,6%
5,6%
3,8%
6,3%
FITC-A (GFP)
Empty Channel

Fig. S3

Fig. S3. Characterization of OE-MSC lines. (A) qRT-PCR of endogenous *Ccr1*, *Ccr3*, *Ccr5*, *Cxcr2*, *Cxcr3*, *Cxcr6* and *Thy* (*Cd90*) expression in mMSCs. Transcript levels are normalized to *Gapdh* expression. Data are presented as mean ± SD from n = 3 independent experiments. **(B)** Lentiviral constructs used to infect mMSCs. Constitutive EF1α and SV40 promoters drive expression of the HA-tagged receptors (i.e. *Ccr1*, *Ccr3*, *Ccr5*, *Cxcr2*, *Cxcr3* and *Cxcr6*, or empty control) and of the eGFP marker respectively. **(C)** qRT-PCR showing expression of the *Ccr1*, *Ccr3*, *Ccr5*, *Cxcr2*, *Cxcr3* and *Cxcr6* genes in the corresponding mMSC-OE lines. Transcript levels are expressed as fold-change to WT-MSC control. Data are presented as mean ± SD from n = 3 independent experiments. Mann-Whitney test was used for statistical analysis. **(D)** Representative immunofluoresce staining of OE-MSC lines (Ccr1, Ccr3, Ccr5, Cxcr2, Cxcr3 and Cxcr6) to verify expression of the eGFP (green) and of the HA (red) tags. **(F)** Representative FACS gating strategy. From left to right: side scatter area vs forward scatter area; forward scatter height vs forward scatter area, to exclude cell-cell aggregates; Pacific Blue area vs side scatter area to exclude $DAPI^+$ and keep only living cells in the analysis; example of an NMDA-damaged retina that had not been transplanted with GFP^+ MSCs, to set gate for GFP^+ cells. **(G)** Representative FACS plots from MSC-WT, MSC-Ccr5, MSC-Cxcr2 and MSC-Cxcr6 transplanted into NMDA-damaged retinae.

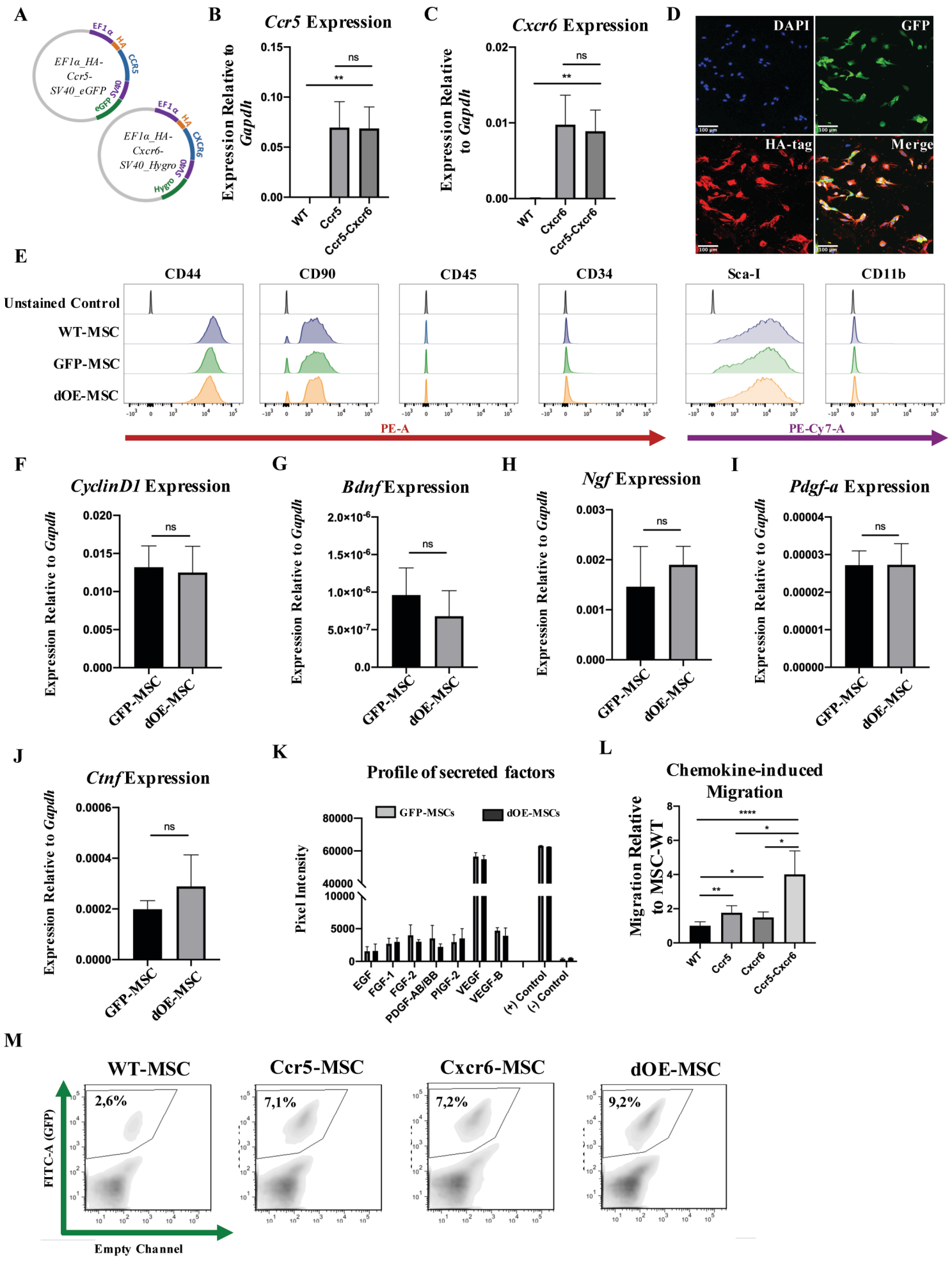
A
EF1α_HA-Ccr5-SV40_eGFP
EF1α_HA-Cxcr6-SV40_Hygro
B
Ccr5 Expression
Expression Relative to Gapdh
WT
Ccr5
Ccr5-Cxcr6
C
Cxcr6 Expression
Expression Relative to Gapdh
WT
Cxcr6
Ccr5-Cxcr6
D
DAPI
GFP
HA-tag
Merge
E
CD44
CD90
CD45
CD34
Sca-I
CD11b
Unstained Control
WT-MSC
GFP-MSC
dOE-MSC
PE-A
PE-Cy7-A
F
CyclinD1 Expression
G
Bdnf Expression
H
Ngf Expression
I
Pdgf-a Expression
J
Ctnf Expression
K
Profile of secreted factors
GFP-MSCs
dOE-MSCs
Pixel Intensity
EGF
FGF-1
FGF-2
PDGF-AB/BB
PlGF-2
VEGF
VEGF-B
(+) Control
(-) Control
L
Chemokine-induced Migration
Migration Relative to MSC-WT
M
WT-MSC
Ccr5-MSC
Cxcr6-MSC
dOE-MSC
2,6%
7,1%
7,2%
9,2%
FITC-A (GFP)
Empty Channel

Fig. S4

Fig. S4. Characterization of Ccr5-Cxcr6-MSCs. (A) Scheme of lentiviral plasmids used to infect MSCs. Constitutive EF1α and SV40 promoters drive expression of the HA-tagged receptors (i.e. *Ccr5* or *Cxcr6*) and of the markers (i.e. eGFP or Hygromycin) respectively. Ccr5-Cxcr6 double positive cells were isolated by FACS-sorting for eGFP and applying hygromycin selection. qRT-PCR showing expression of either the **(B)** *Ccr5* or the **(C)** *Cxcr6* genes in the Ccr5-Cxcr6 double positive MSCs and in the corresponding single-expressing OE-MSC lines (i.e. Ccr5-MSC or Cxcr6-MSC). Transcript levels are expressed as fold-change to GFP^+ Empty- (WT-) MSC control. Data are presented as mean ± SD from n = 3 independent experiments. Two-tailed Student's T-test was used for statistical analysis. **(D)** Representative immunofluoresce staining of Ccr5-Cxcr6-MSCs to verify expression of the eGFP (green) and of the HA (red) tags. Scale bar = 100 μm**. (E)** Flow cytometry analysis comparing the levels of the indicated surface markers (CD44, CD90, CD45, CD34, Sca-I and CD11b) on WT-, GFP- and dOE-MSCs. qRT-PCR showing expression of **(F)** *CyclinD1*, **(G)** *Bdnf,*, **(H)** *Ngf*, **(I)** *Pdgf-α* and **(J)** *Ctnf* genes in GFP- and dOE-MSCs. Transcript levels are normalized to *Gapdh* expression. Data are presented as mean ± SD from n = 3 independent experiments. Two-tailed Student's T-test was used for statistical analysis. **(K)** Profile of neurotrophic factors secreted in SF medium over 24h by GFP- and dOE-MSCs. Pixel intensity is directly proportional to the total amount of protein in sample. No statistically significant changes were detected. Data are presented as mean ± SD from n = 2 independent experiments. **(L)** Quantification of migrated MSCs (Ccr5-, Cxcr6, or Ccr5-Cxcr6) towards a concentration of 50 ng ml^{-1} of Ccl5 and Cxcl16, from transwell-based assays. Number of migrated cells is expressed as fold-change to control (WT-MSC). Data are presented as mean ± SD from n ≥ 3 independent experiments. Mann-Whitney test was used for statistical analysis. **(M)** Representative FACS plots from MSC-WT, MSC-Ccr5, MSC-Cxcr6 and MSC-Ccr5-Cxcr6 transplanted into NMDA-damaged retinae.

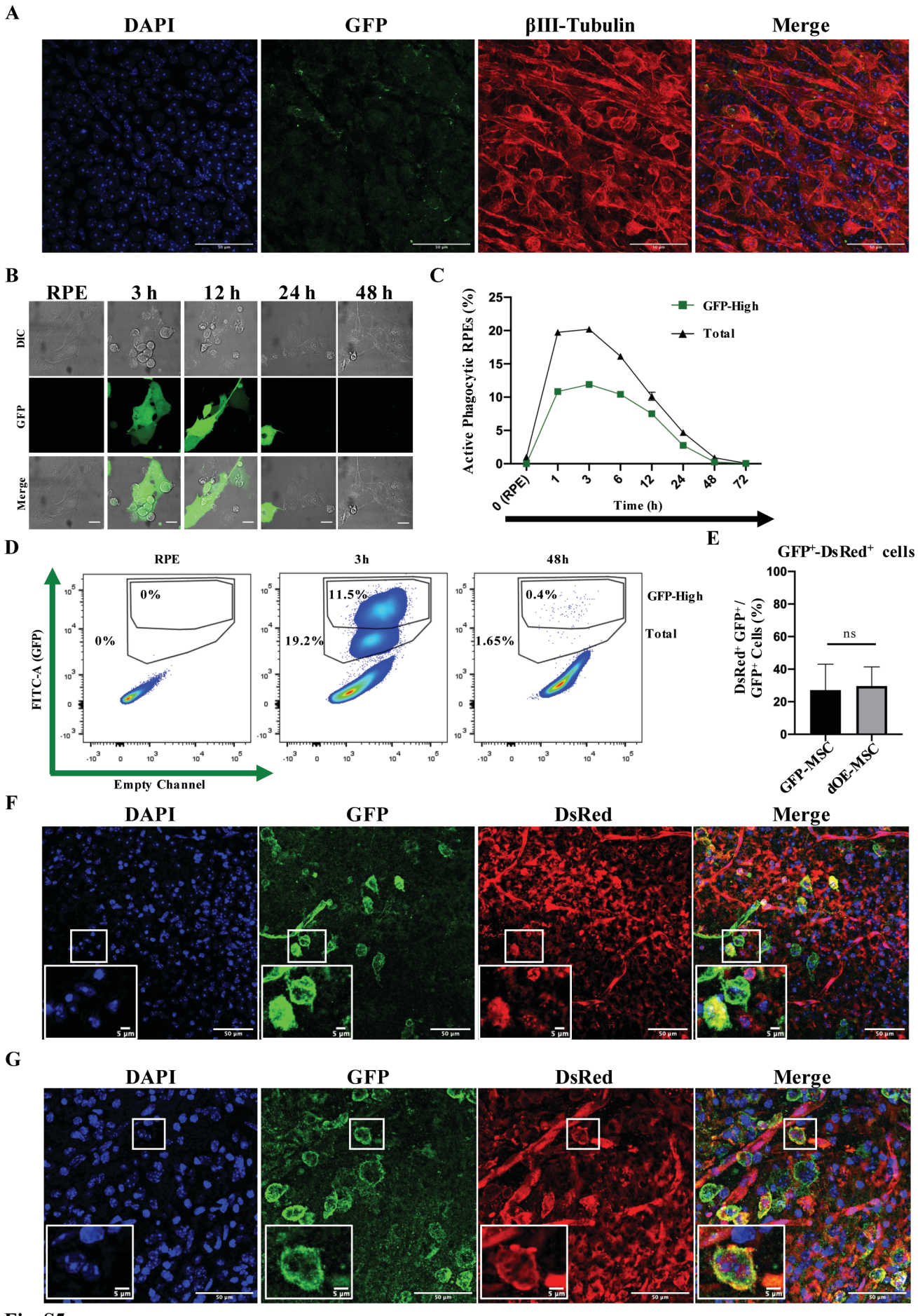
A
DAPI
GFP
βIII-Tubulin
Merge
B
RPE
3 h
12 h
24 h
48 h
DIC
GFP
Merge
C
Active Phagocytic RPEs (%)
GFP-High
Total
0 (RPE)
1
3
6
12
24
48
72
Time (h)
D
RPE
3h
48h
0%
0%
11.5%
19.2%
0.4%
1.65%
GFP-High
Total
FITC-A (GFP)
Empty Channel
E
GFP+-DsRed+ cells
DsRed+ GFP+ / GFP+ Cells (%)
ns
GFP-MSC
dOE-MSC
F
DAPI
GFP
DsRed
Merge
G
DAPI
GFP
DsRed
Merge

Fig. S5

Fig. S5. A percentage of transplanted MSCs might be phagocytosed or undergo cell fusion (A) Field of view from an undamaged area of retinal flat mount prepared from an NMDA-injected eye that had been transplanted with dOE-MSCs, 3 weeks post-injection (3wpi). The tissue was stained for GFP (green), βIII-tubulin (red), and DAPI (blue). Scale bar = 50 μm. **(B)** Imaging of RPE cells co-cultured with GFP-MSC-derived apoptotic bodies for 0 (RPE), 3, 12 and 48h. **(C)** Flow cytometry analysis of RPE cells co-cultured with GFP-MSC-derived apoptotic bodies for 0 (RPE), 1, 3, 6, 12, 48 and 72h. **(D)** Representative FACS plots for RPE (control), 3h and 48h samples. Based on the RPE control, we defined the gate for GFP-positive, actively phagocytic, RPEs (total); within such gate, we also analyzed the population of GFP-high RPE cells (GFP-high). **(E)** Quantification of GFP^+-$DsRed^+$ double positive cells from $DsRed^+$ retinal flat mounts wpi, expressed as a percentage over the total GFP^+ cells in the field of view. Data are presented as mean ± SD (n = 3). Two-tailed Student's T-test was used for statistical analysis. **(F, G)** Representative field of view from retinal flat mounts prepared from an NMDA-damaged $DsRed^+$ eye that had been transplanted with dOE-MSCs (3wpi). The tissue was stained for GFP (green), DsRed (red) and DAPI (blue). Scale bar = 50 μm. Inserts at the bottom-left corner show higher magnification of the regions in the white square and include one representative heterokaryon (scale bar = 5 μm).

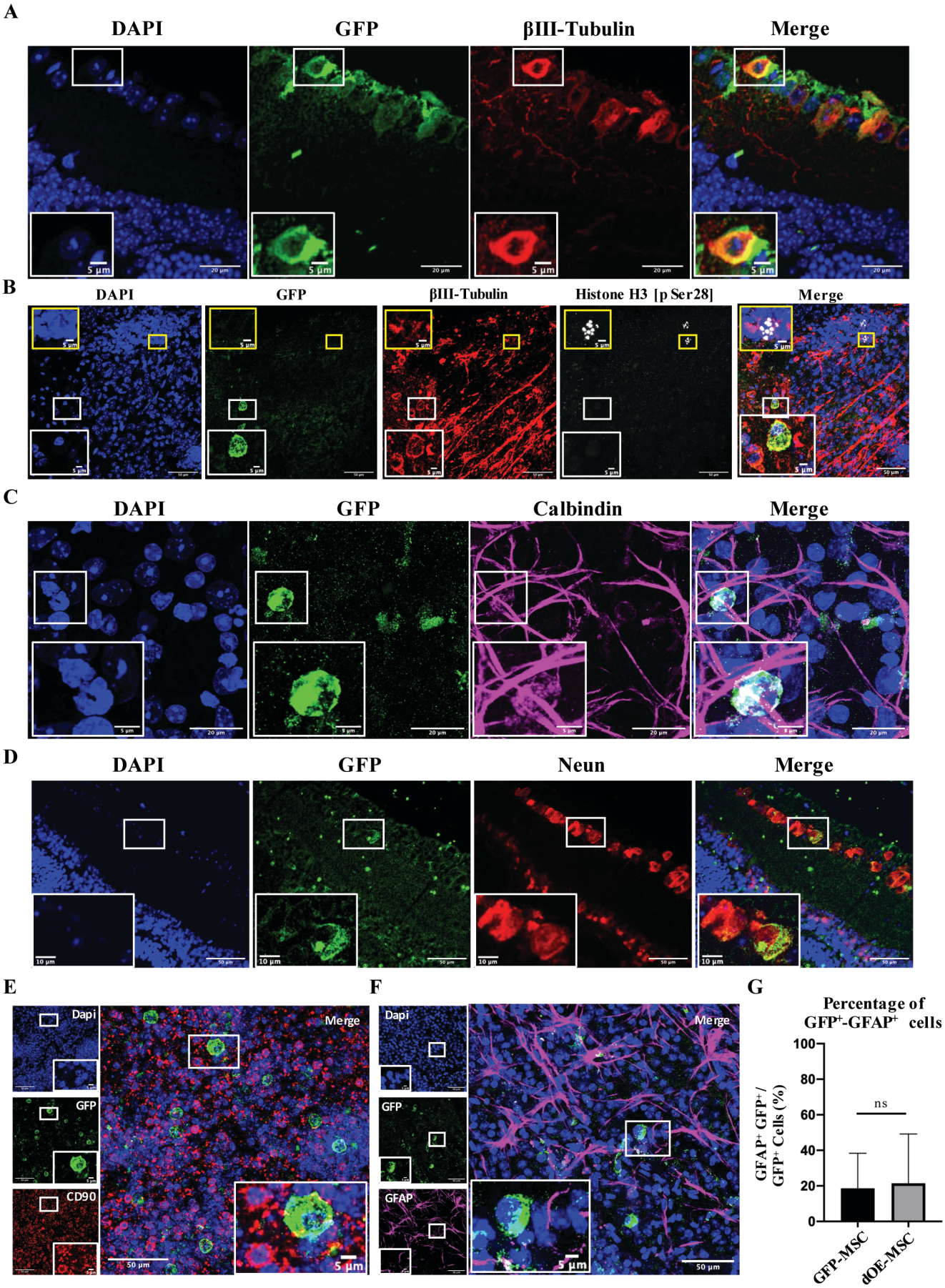
A
DAPI
GFP
βIII-Tubulin
Merge
B
DAPI
GFP
βIII-Tubulin
Histone H3 [p Ser28]
Merge
C
DAPI
GFP
Calbindin
Merge
D
DAPI
GFP
Neun
Merge
E
Dapi
GFP
CD90
Merge
F
Dapi
GFP
GFAP
Merge
G
Percentage of GFP+-GFAP+ cells
GFAP+ GFP+ / GFP+ Cells (%)
ns
GFP-MSC
dOE-MSC

Fig. S6

Fig. S6. Transplanted MSCs integrate into the NMDA-damaged retina and express neural markers 3 weeks post-injection. (A) Representative retinal section prepared from an NMDA-damaged eye that had been transplanted with dOE-MSCs, 3wpi. The tissue was stained for GFP (green), βIII-tubulin (red) and DAPI (blue). Scale bar = 20 μm. Inserts at the bottom-left corner show higher magnification of the regions in the white square (scale bar = 5 μm). **(B)** Representative field of view from retinal flat mounts prepared from an NMDA-damaged eye that had been transplanted with dOE-MSCs (3wpi). The tissue was stained for GFP (green), βIII-tubulin (red), phospho-Histone H3 (Ser10) (white) and DAPI (blue). Scale bar = 50 μm. Inserts at the bottom-left and upper-right corners show higher magnification of the regions in the white and yellow squares respectively (scale bar = 5 μm). **(C)** Representative field of view from retinal flat mounts prepared from an NMDA-damaged eye that had been transplanted with dOE-MSCs (3wpi). The tissue was stained for GFP (green), calbindin (magenta) and DAPI (blue). Scale bar = 20 μm. Inserts at the bottom-left corner show higher magnification of the regions in the white square (scale bar = 5 μm). **(D)** Representative retinal section prepared from an NMDA-damaged eye that had been transplanted with dOE-MSCs, 3wpi. The tissue was stained for GFP (green), Neun (red) and DAPI (blue). Scale bar = 50 μm. Inserts at the bottom-left corner show higher magnification of the regions in the white square (scale bar = 10 μm). **(E)** Representative field of view from retinal flat mounts prepared from an NMDA-damaged eye that had been transplanted with dOE-MSCs (3wpi). The tissue was stained for GFP (green), CD90 (red) and DAPI (blue). Scale bar = 50 μm. Inserts at the bottom-right corner show higher magnification of the regions in the white square (scale bar = 5 μm). **(F)** Representative field of view from retinal flat mounts prepared from an NMDA-damaged eye that had been transplanted with dOE-MSCs (3wpi). The tissue was stained for GFP (green), GFAP (magenta) and DAPI (blue). Scale bar = 50 μm. Inserts at the bottom-left corner show higher magnification of the regions in the white square (scale bar = 5 μm). **(G)** Quantification of GFP^+-$GFAP^+$ double positive cells from NMDA-damaged retinal flat mounts 3wpi, expressed as a percentage over the total GFP^+ cells in the field of view. Data are presented as mean ± SD (n = 3). Two-tailed Student's T-test was used for statistical analysis.

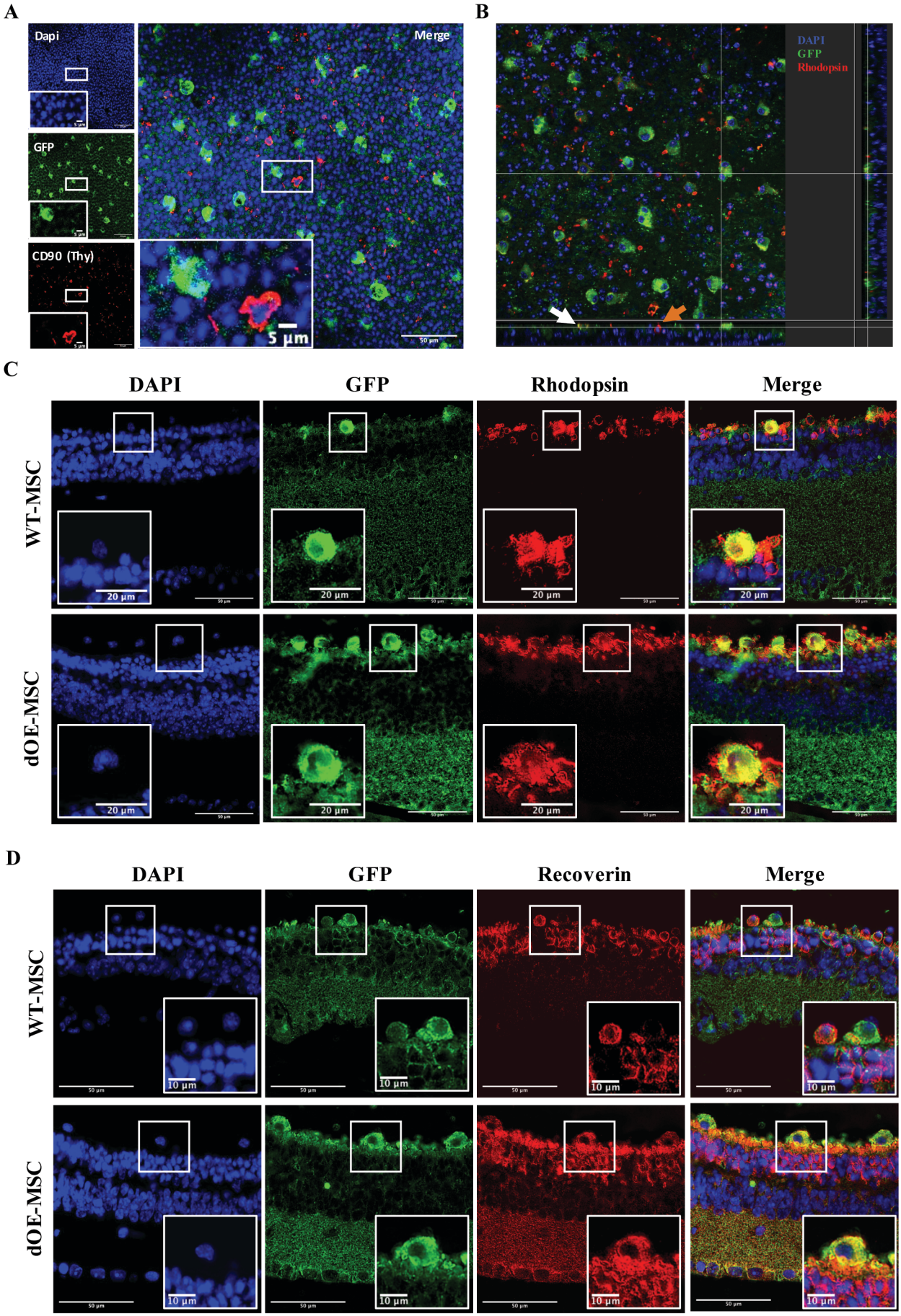
A
Dapi
Merge
GFP
CD90 (Thy)
5 μm
B
DAPI
GFP
Rhodopsin
C
DAPI
GFP
Rhodopsin
Merge
WT-MSC
dOE-MSC
20 μm
D
DAPI
GFP
Recoverin
Merge
WT-MSC
dOE-MSC
10 μm

Fig. S7

Fig. S7. Transplanted MSCs partially integrate into the host tissue and express photoreceptor markers 3 weeks post-injection. (A) Representative field of view from retinal flat mounts prepared from an *rd10* eye that had been transplanted with dOE-MSCs (3wpi). The tissue was stained for GFP (green), CD90 (red) and DAPI (blue). Scale bar = 50 μm. Inserts at the bottom-left corner show higher magnification of the regions in the white square (scale bar = 5 μm). **(B)** Orthogonal view from a retinal flat mount that had been transplanted with dOE-MSCs, 3wpi. The tissue was stained for GFP (green), rhodopsin (red), and DAPI (blue). The white arrow points at a GFP^+ cells that appears to be physically located within the same layer as the $rhodopsin^+$ (orange arrow). **(C, D)** Representative retinal section prepared from an *rd10* eye that had been transplanted with either GFP- (up) or dOE- (down) MSCs, 3wpi. The tissue was stained for GFP (green), rhodopsin **(C)** or recoverin **(D)** (red), and DAPI (blue). Scale bar = 50 μm. Inserts at the bottom corner show higher magnification of the regions in the white square (scale bar = 20 μm - C - or 10 μm - D).

Gene	Primer FW	Primer RV
mIl1-β	gccacctttgacagtgatgaga	ggacagcccaggtcaaaggt
mCcl5	tgcagaggactctgagacagc	gagtggtgtccgagccata
mCcl6	tctttatccttgtggctgtcc	tggagggttatagcgacgat
mCcl12	ggtattggctggaccagatg	gggacactggctgcttgt
mCxcl1	agactccagccacactccaa	tgacagcgcagctcattg
mCxcl2	actccagactccagccacac	cagttcactggccacaacag
mCxcl5	tcttgggtgtgttaagagtgttct	cacagcagctttctaaaaccataa
mCxcl9	ccatgaagtccgctgttctt	tgagggatttgtagtggatcg
mCxcl10	atcagcaccatgaacccaag	ttccctatggccctcattct
mCxcl11	gcggctgctgagatgaac	cgcccctgtttgaacataag
mCxcl16	tgaactagtggactgctttgagc	gcaaatgtttttggtggtga
hIL1-β	tacctgtcctgcgtgttgaa	tctttgggtaatttttgggatct
hTNF-α	cagcctcttctccttcctgat	gccagagggctgattagaga
hCCL2	agtctctgccgcccttct	gtgactggggcattgattg
hCCL5	cgctgtcatcctcattgcta	ggtgtggtgtccgaggaata
hCCL21	tccatcccagctatcctgtt	agctcctttgggtctgcac
hCCL22	cgtggtgaaacacttctactgg	ccttatccctgaaggttagcaa
hCCL23	ccaggaggatgaaggtctcc	catcatgaactctgtctctgcat
hCXCL1	tcctgcatcccccatagtta	cttcaggaacagccaccagt
hCXCL2	cccatggttaagaaaatcatcg	cttcaggaacagccaccaat
hCXCL3	aaatcatcgaaaagatactgaacaag	ggtaagggcagggaccac
hCXCL6	gtccttcgggctccttgt	cagcacagcagagacaggac
hCXCL9	tgttccccttttgcttcattc	gaaaggcactgcattgtgg
hCXCL10	gaaagcagttagcaaggaaaggt	gacatatactccatgtagggaagtga
hCXCL11	agtgtgaagggcatggcta	tcttttgaacatggggaagc
hCXCL16	gccctttcctatgtgctgtg	caggtatataatgaaccggcagat
mCcr1	ctgtgtggacaaaatactctgga	tggggtaggcttctgtgaaa
mCcr3	gaatcaaagagctggggtca	caggaggccgatgatgaa
mCcr5	caactttggggtgataacaagtg	tggtaaagattatttctgggagaga
mCxcr2	caggaccaggaatgggagta	tcccctccaaatatccccta
mCxcr3	gtggccaagtcagtcacctc	cccacaaaggcatagagcag
mCxcr6	ccagctttaagtatgccatcg	ttaaggcaagcccgaaagta
mCyclinD1	gagattgtgccatccatgc	ctcctccttcgcacttctgct

mBDNF	cagtggacatgtccggtgggacggtc	ttcttggcaacggcaacaaaccacaac
mNGF	tatactggccgcagtgaggt	ggacattgctatctgtgtacgg
mPDGFa	gatgaggacctgggcttg	gatcaactcccggggtatct
mCTNF	gacctgactgctcttatggaatct	gcctggaggttctcttgga

Table S1. Primers used for qRT-PCR analysis.

Compound Name	**Catalogue Number (R&D Systems)**	**Receptor Inhibited**	**Working Concentration**
J 113863	2595/10	Ccr1	6 μM
SB 328437	3650/10	Ccr3	25 μM
Maraviroc	3756/10	Ccr5	7 μM
SB 332235	5671/10	Cxcr2	8 μM
ML 339	5943/10	Cxcr6	18 μM

Table S2. Name, catalogue number and working concentration of selective receptor antagonists.

Gene	**Primer FW**	**Primer RV**
mCcr1-CDS	atggagatttcagatttcacagaag	tcagaagccagcagagagc
mCcr3-CDS	atggcattcaacacagatgaaatc	ctaaaacaccacagagatttcttg
mCcr5-CDS	atggattttcaagggtcagttcc	tcataaaccagtagaaacttcatg
mCxcr2-CDS	atgggagaattcaaggtggataag	ttagagggtagtagaggtgtttg
mCxcr3-CDS	atgtaccttgaggttagtgaacgt	ttacaagcccaggtaggagg
mCxcr6-CDS	atggatgatgggcatcaagagtc	ctacaattggaacatactggtggtc

Table S3. List of primers used to amplify receptors' CDSs from total mouse spleen cDNA.