## Supplementary Appendix:

## Morphine promotes neovascularizing retinopathy in sickle transgeneic mice

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## Methods

### Adenosine diphosphatase (ADPase) staining of whole retinal blood vessels

We enabled visualization of retinal vessels by detecting their ADPase activity, as described in detail previously.[1] Briefly, after eyes were enucleated from euthanized mice, retinae were fixed in 2% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.2), followed by 20 hr incubation, washing, and permeabilization using Triton X-100. To detect ADPase, retinae were incubated in buffer that included 3 mM lead nitrate and 1mg/ml ADP In darkfield illumination examination of retinae flat mounts, the ADPase-positive retinal vessel were clearly defined as white due to the lead ADPase reaction product. Images were recording with a digital camera using DVC acquisition software version 2.28 (DVC Corporation, Austin, TX).

### Imaging and scoring of retinal neovascularization

Using Photoshop 6.0), a high pass filter was applied to sharpen edge detection of the retinal vasculature. Images were then thresholded at the same tonal value and then skeletonized using a Photoshop plugin, the Image Processing Toolkit (RGI, Raleigh, NC). Interference from the skeletonized edge surrounding the retina was removed manually using an erase tool, and then the length, nodes (branch points) and end points (number) were measured to provide numerical representations of angiogenesis robustness, as we described previously.[2]

**Mouse retinal endothelial cells (REC) isolation and culture**

Primary cell cultures of REC were established as described previously.[3] Briefly, retinae from 3-month-old WT or NY1DD mice were excised aseptically under a dissecting microscope, digested using trypsin, transferred to a single fibronectin coated well of a 24-well plate, minced, and incubated with microvascular endothelial cell culture medium.[3] After 1-2 weeks of culture, RECs were selected by panning using anti-CD31 antibodies and propagated. Only REC passages 4-6 were used for experiments.

Cultures were checked for purity by: immunofluorescent staining for CD31, VWF, and FLK1 (VEGF-R2, LY-73); uptake of acetylated low-density lipoprotein; and FACS identification of CD31 and FLK1.[2, 3]

**Cell Membrane preparation**

RECs from WT and NY1DD mice were harvested and washed twice with phosphate buffer solution (PBS). Cells were then sedimented by centrifugation (10 min; 300 x g). Cell pellets were homogenized in a sucrose-HEPES solution (0.32 M sucrose, 10 mM HEPES, pH 7.7) with a glass Wheaton Dounce homogenizer (Kontes Glass Co., Vineland, NJ). After centrifuging at 1000 x g for 10 min to remove unbroken cells and nuclei, membranes were isolated by centrifugation at 100,000 x g for 60 min. Membrane pellets were resuspended in the sucrose-HEPES solution and protein concentration was determined.[4] All steps were performed at 4°C.

**Radio-ligand Binding**

Saturation binding experiments were performed at several concentrations (0.078 -10 nM) of [$^3$H] diprenorphine (Perkin Elmer), with a constant specific activity of 50 Ci/mmol.[5] Nonspecific binding was assessed in parallel assays in presence of 10 μM naloxone (Hospira Inc., Lake Forest, Il).

Competition binding assays were performed in the presence of a constant final concentration of [$^3$H] diprenorphine (2 nM) and varying concentrations ($10^{-11}$-$10^{-5}$ M) of morphine (Baxter Esilerderle Mfd. Healthcare Corporation), etorphine ([D-Ala$^2$, D-Leu$^5$]enkephalin; National Institute on Drug Abuse), DAMGO (Tyr-D-Ala-Gly-N-methyl-Phe-Gly-ol; Sigma), DPDPE ([D-Pen2,5]-Enkephalin acetate hydrate; Sigma), or U50488H (Tocris). For each ligand, three separate assays were performed, each in triplicate.

Specific binding was defined as the difference between total and nonspecific binding in the presence of 10 μM naloxone. Data were analyzed using Graph Pad Prism software (Version 4.0), to obtain the affinity constant ($K_d$), receptor density ($B_{max}$), and inhibition constant ($K_i$) values for each ligand. In saturation binding assays, data were fit to the non-linear function and to the linear transformation (Scatchard plot; Bound/Free versus Bound).

**Cell proliferation assay**

REC were plated in complete culture medium, and after 24 h they were switched to serum-free and growth factor-depleted medium overnight, before 48 h incubation with morphine or $VEGF_{164}$ (R&D Systems). Opioid receptor-specific proliferation assays used morphine (1 μM), DAMGO (1 μM), DPDPE (1 μM), and U50488H (1 μM), naolxone (1 μM) and CTOP (1 μM, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH2; Tocris) with cell proliferation measured using BrdU (colorimetric) cell proliferation ELISA assay (Roche Diagnostics) as described previously.[2, 3]

**Apoptosis assay**

REC were cultured in a medium depleted of serum and growth factor and incubated with 10μM morphine or 100 ng/ml $VEGF_{164}$ for 48 h, followed by determination of cytoplasmic histone-associated-DNA-fragments (mono- and oligonucleosomes) using Cell Death Detection ELISA kit (Roche Diagnostics).[6]

**IL6 and TNFα levels in serum and REC conditioned medium**

Serum was collected from WT and NY1DD mice treated with morphine or PBS for 15 months as described under "Drug treatment" and frozen at -80°C until use. RECs ($1x10^6$/well) in 6 well plates were incubated in complete culture medium for 24 h and then cultured overnight in medium depleted of serum and growth factors. Cells were incubated with morphine (1 μM) for 48 h, before cell-free conditioned medium was collected. IL6 and TNFα levels in both serum and REC conditioned medium were measured by ELISA according to the manufacturer's instructions (R&D systems) using a microplate reader (Synergy HT, Biotek, Winooski, VT) and calculated with the plate reader Gen5™ 1.0 data analysis software (Biotek). All analyses and calibrations were performed in triplicate, with 3 replicate samples per condition with appropriate controls.

**RNA Isolation and RT-PCR**

Unfixed retinas or REC grown for 48h in complete culture medium were harvested for isolation of total RNA. For evaluation of Stat3 signaling on receptor expression, REC grown for 48h in complete culture medium were serum- and growth factor-depleted overnight. Cells were then incubated for 48h in basal media containing 10 μM morphine or IL6 (1 ng/ml; R&D Systems) with or without STAT3 inhibitor peptide (PpYLKTK-mts , 0.5 mM; Calbiochem, La Jolla, CA) and harvested for isolation of total RNA. Total RNA was isolated with Trizol reagent (Invitrogen, Carlsbad, CA) and 5 μg total RNA was reverse transcribed using the first strand synthesis system (Invitrogen). PCRs were performed by using Taq DNA polymerase (Continental Lab Products, San Diego, CA).

Sequences of primers homologous to coding region of each gene were:

<u>mouse MOR (accession number NM_011013)</u>

sense nucleotide 99-118: 5'-CGA CTG CTC TGA CCC CTT AG-3'

antisense nucleotides 302-321: 5'-TCC AAA GAG GCC CAC TAC AC-3'

<u>mouse VEGFR2/Flk1/KDR (accession number NM_010612)</u>

sense nucleotide: 5’ TGG GAA ACC TGC AAG CAA AAT 3’

anti-sense nucleotide: 5’TTGGAGGACAGAGCCACTGTTTGA 3’

mouse GAPDH (accession number NM_13985769)

sense nucleotide 198-212: 5’-CGT CTT CAC CAC CAT GGA GA-3’

antisense nucleotide: 5’-CGG CCA TCA CGC CAC AGT TT-3’

Amplification was performed for 30 cycles at 94°C for 50s, 56°C for 50s and 72°C for 50s, with a final extension cycle for 10 min at 72° in PTC-100 Thermocycler (MJ Research, Waltham, MA). PCR products obtained were sequenced (Microchemical Facility, University of Minnesota) to confirm matching with the expected DNA sequences. DNA samples were visualized by 2% agarose gel electrophoresis. GAPDH was used for normalization and relative gene expression was calculated against the normalized value for PBS-treated WT mice.

## Supplementary References

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3. Chen C, Farooqui M, Gupta K. Morphine stimulates vascular endothelial growth factor-like signaling in mouse retinal endothelial cells. Curr Neurovasc Res. 2006;3(3):171-180.
4. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem. 1951;193(1):265-275.
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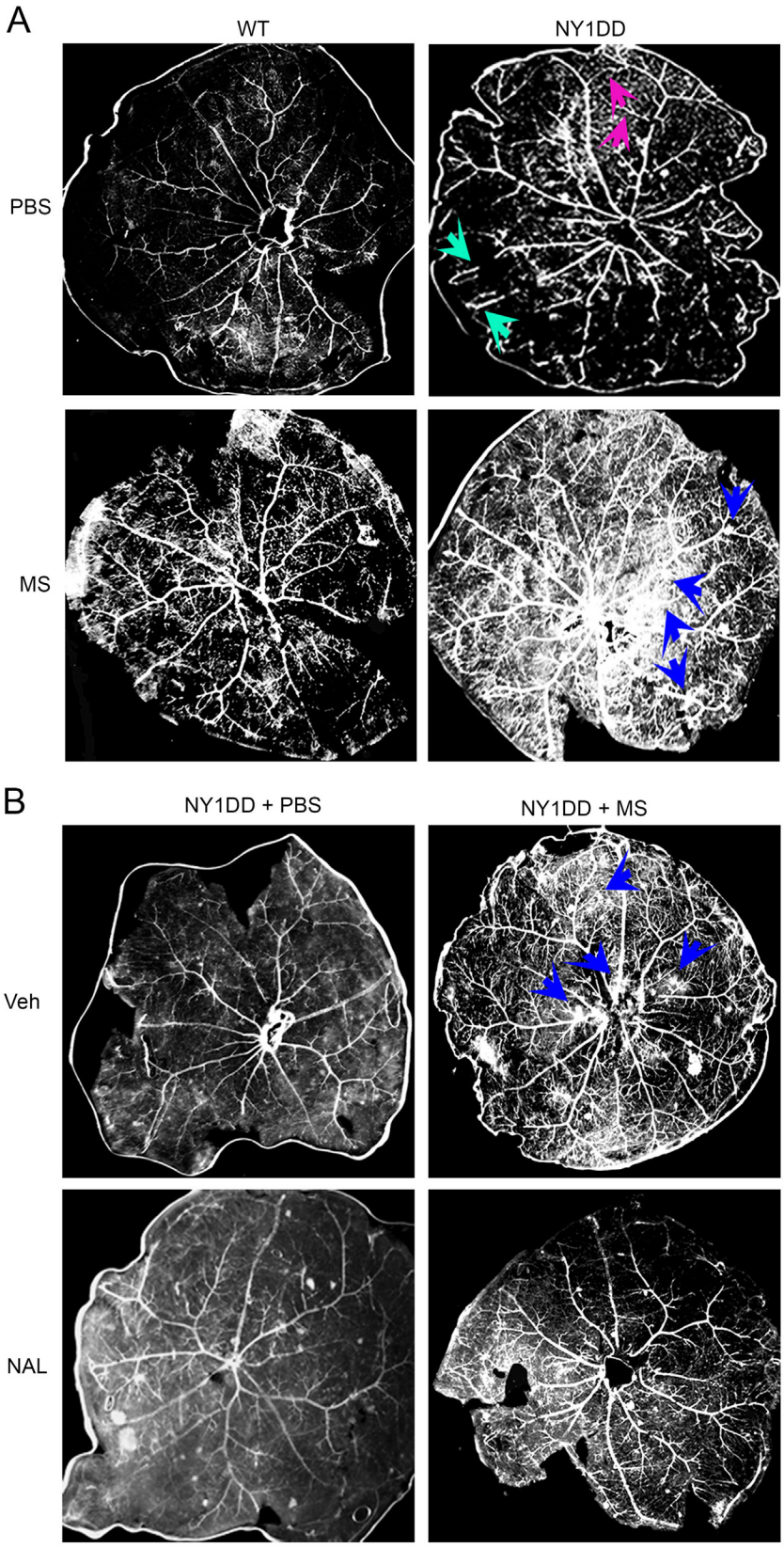
6. Gupta K, Kshirsagar S, Li W, et al. VEGF prevents apoptosis of human microvascular endothelial cells via opposing effects on MAPK/ERK and SAPK/JNK signaling. Exp Cell Res. 1999;247(2):495-504.

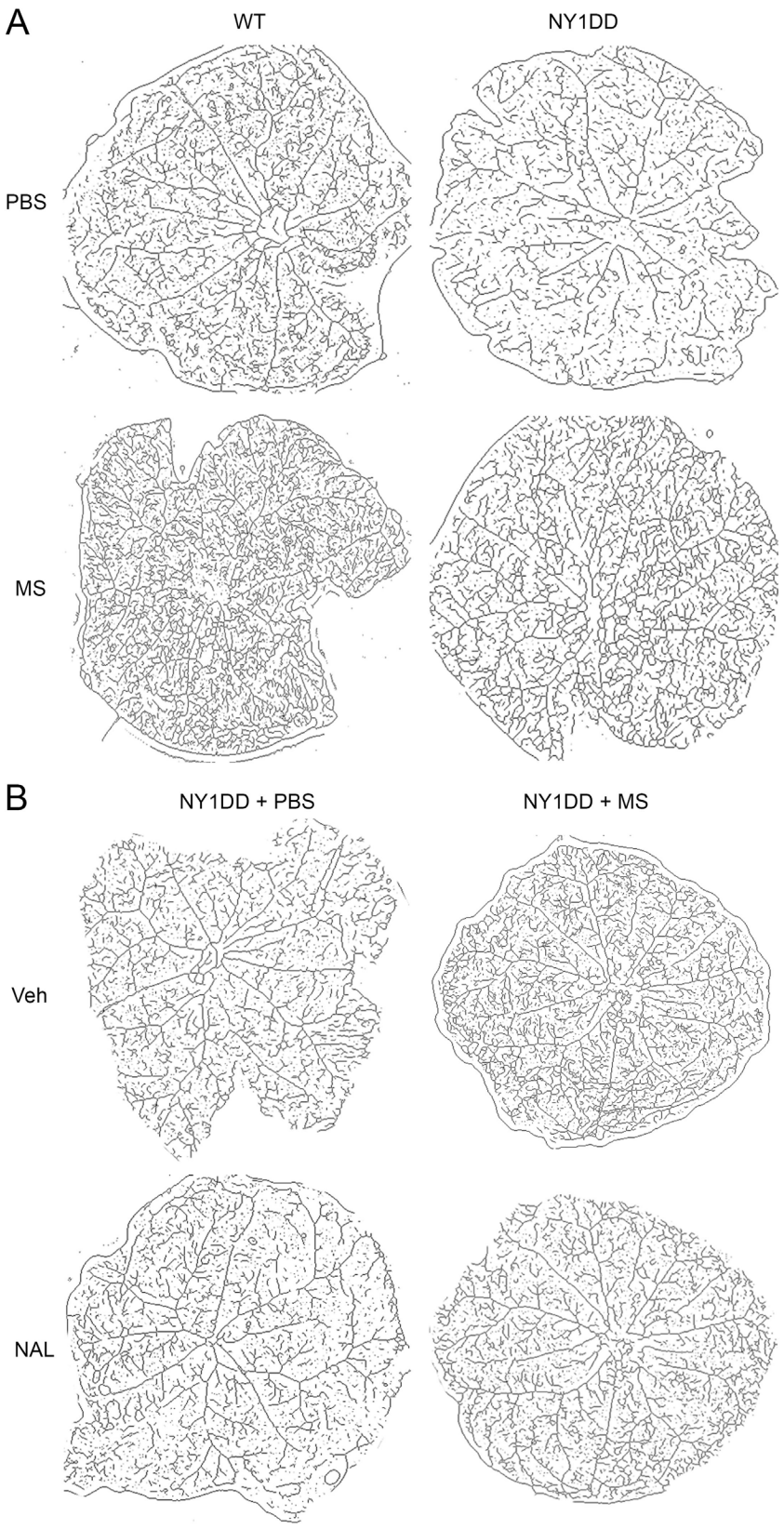
## Legends to Supplementary Figures

**Supplementary Figure S1.** *Morphine induces retinal neovascularization in sickle mice, which is attenuated by an opioid receptor antagonist, naloxone*. (A) C57BL/6 mice (WT) or NY1DD sickle mice treated with PBS or increasing doses of morphine (MS) for 15 months and (B) NY1DD sickle mice treated with MS alone, MS with naloxone (MS + NAL) or PBS (Veh) or naloxone (NAL) for 10 months. (A, B) Representative images of isolated retinae with ADPase staining of blood vessels (Magnification 400X). Retinae of NY1DD sickle mice treated with PBS had typical hairpin loops in the periphery (green arrows) and few arterio-venous anastamosis (magenta arrows). Morphine-treated NY1DD sickle mice had more arterio-venous anastamosis (blue arrows), while the treatment with naloxone resulted in a more normal vascular pattern. MS, morphine sulfate; Nal, naloxone; PBS, phosphate buffered saline; Veh, vehicle (saline).

**Supplementary Figure S2.** *Digital skeletonization of ADPase stained retina.* Skeletonized and linearized digital images showing the blood vessel length, nodes (branch points) and ends (number of blood vessels*)*. (A) C57BL/6 mice (WT) or NY1DD sickle mice treated with PBS or increasing doses of morphine (MS) for 15 months and (B) NY1DD sickle mice treated with MS alone, MS with naloxone (MS + NAL) or PBS (Veh) or naloxone (NAL) for 10 months MS, morphine sulfate; Nal, naloxone; PBS, phosphate buffered saline; Veh, vehicle (saline).




**Supplementary Figure S1**




**Supplementary Figure S2**