Supplemental Data:
"Nitric Oxide Synthase is not essential for *Drosophila* development"

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Supplemental Experimental procedures

Strains

PBac{WH}Nos^{f02469} and *PBac{RB}Nos^{e02671}* were obtained from the Exelexis Collection at the Harvard Medical School. *Nos^{C}* was kindly provided by G. Enikolopov. To generate the *Nos$^{\Delta 15}$* deletion, *hs-Flp; PBac{WH}Nos^{f02469}/ PBac{RB}Nos^{e02671}* were heat-shocked and crossed to a balancer stock; progeny that were homozygous for candidate recombinant chromosomes were screened by PCR. Flies carrying the deletion were then out-crossed to *w^{1118}* for at least five generations.

To remove the lethality associated with *Nos^{C}*, *Nos^{C}/PBac{WH}Nos^{f02469}* females were crossed to *Nos^{C}/CyO* males and viable, non-CyO progeny were screened for lack of the *white* transgene associated with *PBac{WH}Nos^{f02469}*. Out of ~7500 progeny screened, we isolated three lines carrying candidate recombinant chromosomes (recombination frequency = [(3 X 2)/ 7500] X 100 = 0.08%). The physical distance was calculated using an estimated recombination frequency of 3.6 m.u. (map units) per Mb for this region [S1]: 0.08 m.u./3.6 m.u. per Mb = 0.022Mb. Candidates were tested for retention of the glycine to glutamic acid substitution by sequencing.

PCR and rtPCR

PCR of recombinants was performed with the following primers:
PCR A_forward: ATTTGAGAACCTGCGATTCGTG
PCR A_reverse: TGCCCTCGATGTTCTTCAGTCTC
PCR B_forward: TTCTGATTTTGAAGGCATAGTTTTC
PCR B_reverse: GCAGTTAGAGGTTAGACAAGTGCTC
control_forward: TGTTGTGCAGGAACTAAACAATG
control_reverse: GATTGGGACCCGTTACTTAAATC

RNA for rtPCR was extracted from *w^{1118}* and *Nos$^{\Delta 15}$* using TRIzol Reagent (Invitrogen, cat: 15596-018) according the manufacturer's instructions and cDNA was generated using Superscript reverse transcriptase II (Invitrogen, cat: 18064-014). Primers used for the subsequent PCR are as follows:
Exon 2_forward: GATGTCGCAGCATTTCACATCGATATT
Exon 5_reverse: GTTTCCATCGCGTCTCGTGGGC
Exon 5_forward: GGCTCGTCAAAGTCCTTCATGAAG
Exon 8_reverse: CAAAGTAGTCCGGATCGTGAC
Exon 8_forward: GTCACGATCCGGACTACTTTG
Exon 9_reverse: CATCGAACAGCATACTGGATAC
Exon 15_forward: GGCTCGTCAAAGTCCTTCATGAAG
Exon 18_reverse: CCGCAGTGTTAGCAAAAATGTC

NOS activity assays and western blotting

NOS activity was measured using L-[2,3,4,5-^{3}H]Arginine (GE Healthcare, cat: TRK698) and the NOS Activity Assay Kit (Cayman Chemical Company, cat: 781001) according to the manufacturer's instructions with the following modifications: 40 heads of each genotype were homogenized in 50uL buffer using a Teflon pestle. An aliquot of extract was reserved for western analysis, and 10uL of extract was incubated with 40uL reaction mix for 4 hours at 29C. 7mM L-valine was added to the reaction mix to inhibit potential arginase activity, which would interfere with the analysis; L-NNA was used to inhibit NOS activity in extracts from w^{1118}. 3uL of each reaction was subjected to thin layer chromatography to separate arginine (R_f of approximately 0.4) from citrulline (R_f of approximately 0.9) using solvent consisting of chloroform, methanol, ammonium hydroxide and water (1:9:4:2). [^{3}H] decay was measured by liquid scintillation. NOS activity is represented by cpm at the citrulline spot as a percent of the total cpm recovered (citrulline plus arginine). Significance was determined by the Students' T-test. Rabbit anti-NOS antibody was raised against a peptide corresponding to the entirety of exon 16, which is in the C-terminal reductase domain, fused to a 6X-Histidine tag. Antibodies were used under the following conditions: Rabbit anti-NOS (1/400), mouse anti-β-tubulin (1/10000, Molecular probes), HRP anti-rabbit and HRP anti-mouse (1/5000, Jackson Laboratories).

Diaphorase activity staining

Diaphorase staining was performed essentially as in [S2]. In short, larvae were dissected in 4% formaldehyde and fixed in 4% formaldehyde in PBS with 0.2% Triton x-100 over night. After washing, tissue was stained with fresh 0.2mM b-NADPH and 0.2 mM nitroblue tetrazolium (NBT) for 1-3 hours.

Supplemental References:

S1. *Drosophila melanogaster* Recombination Rate Calculator [Internet]. Stanford University (USA): Fiston-Lavier, A., and Petrov, D.; c2007 [modified 2008 September; cited 2009 December].
Available at http://petrov.stanford.edu/cgi-bin/recombination-rates_updateR5.pl

S2. Ott, S., and Elphick, M. (2003). New Techniques for Whole-mount NADPH-diaphorase Histochemistry Demonstrated in Insect Ganglia. J. Histochem. Cytochem. *51*, 523-532.