1 *Full Title:*

2 **Nitric Oxide Mediates Neuro-Glial Interaction that Shapes *Drosophila* Circadian Behavior**

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4 *Short Title:*

5 Nitric Oxide and *Drosophila* Circadian Rhythms

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7 ***Authorship and Affiliations:***

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Abstract

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19 *Drosophila* circadian behavior relies on the network of heterogeneous groups of clock neurons.

20 Short- and long-range signaling within the pacemaker circuit coordinates molecular and neural

21 rhythms of clock neurons to generate coherent behavioral output. The neurochemistry of

22 circadian behavior is complex and remains incompletely understood. Here we demonstrate that

23 the gaseous messenger nitric oxide (NO) is a signaling molecule linking circadian pacemaker to

24 rhythmic locomotor activity. We show that two independent mutants lacking nitric oxide

25 synthase (NOS) have severely disturbed locomotor behavior both in light-dark cycles and

26 constant darkness, although molecular clocks in the main pacemaker neurons are unaffected.

27 Behavioral phenotypes are due in part to the malformation of neurites of the main pacemaker

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28   neurons, s-LNvs. Using cell-type selective and stage-specific gain- and loss-of-function of NOS,

29   we demonstrate that NO secreted from diverse cellular clusters non-cell-autonomously affect

30   molecular and behavioral rhythms. We further identify glia as a major source of NO that

31   regulates circadian locomotor output. These results reveal for the first time the critical role of

32   NO signaling in *the Drosophila* circadian system and highlight the importance of neuro-glial

33   interaction in the neural circuit output.

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Author summary

37   Circadian rhythms are daily cycles of physiological and behavioral processes found in most

38   plants and animals on our planet from cyanobacteria to humans. Circadian rhythms allow

39   organisms to anticipate routine daily and annual changes of environmental conditions and

40   efficiently adapt to them. Fruit fly *Drosophila melanogaster* is an excellent model to study

41   this phenomenon, as its versatile toolkit enables the study of genetic, molecular and

42   neuronal mechanisms of rhythm generation. Here we report for the first time that

43   gasotransmitter nitric oxide (NO) has a broad, multi-faceted impact on *Drosophila*

44   circadian rhythms, which takes place both during the development and the adulthood. We

45   also show that one of the important contributors of NO to circadian rhythms are glial cells.

46   The second finding highlights that circadian rhythms of higher organisms are not simply

47   controlled by the small number of pacemaker neurons but are generated by the system

48   that consists of many different players, including glia.

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Introduction

53   Our environment undergoes daily fluctuations in solar illumination, temperature, and other

54   parameters. Organisms across the phylogenetic tree are equipped with circadian clocks, which

55   help predict daily environmental changes and create temporal patterns of behavioral and

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56   physiological processes in concordance with the environmental cycle. *Drosophila melanogaster*

57   remains a handy model to study this phenomenon ever since Konopka and Benzer identified the

58   first clock gene, *period*, in this organism (1).

59   *Drosophila* circadian clocks rely on transcriptional-translational feedback loops that

60   operate using an evolutionarily conserved principle. In the main loop, CLOCK/CYCLE

61   (CLK/CYC) heterodimers bind to the E-boxes in the promoter regions of the *period* (*per*) and

62   *timeless* (*tim*) genes and activate their transcription. PER and TIM proteins undergo post-

63   translational modifications and enter the nucleus to suppress their own production by inhibiting

64   CLK/CYC activity. CLK/CYC also activates transcription of the genes encoding the basic-zipper

65   regulators PAR DOMAIN PROTEIN 1 (PDP-1) and VRILLE (VRI), which activates and

66   inhibits *Clk* gene expression, respectively. Thus, positive- and negative- feedback loops created

67   by PDP-1 and VRI with CLK/CYC are interlocked with the main negative-feedback loop and

68   ensure the generation of 24-h rhythms (2,3).

69   In the fly brain, molecular clocks are present in ca.150 so-called clock neurons, which

70   form the pacemaker circuit controlling circadian behavior. Clock neurons are classified into

71   groups according to their morphological characteristics and location: small and large lateral

72   ventral neurons (s- and l-LNvs), lateral dorsal neurons (LNds), lateral posterior neurons (LPNs)

73   and three groups of dorsal neurons (DN1s, DN2s, DN3s) (4,5). Although all clock neurons

74   express a common set of clock genes, they are heterogeneous in terms of

75   neurotransmitter/neuropeptide phenotype, function, and composition of the molecular clock.

76   Neuropeptide pigment-dispersing factor (PDF) is uniquely secreted from the l-LNvs and 4 out of

77   5 s-LNvs. Several other neuropeptides, including small neuropeptide F (sNPF) and ion transport

78   peptide (ITP), and classical neurotransmitters such as glutamate and glycine, are also expressed

79   across pacemaker circuit (6,7). PDF-positive s-LNvs are designated as the Morning (M)

80   oscillator, whereas LNds together with the PDF-negative 5th s-LNv consist of the Evening (E)

81   oscillator. Under the light-dark (LD) experimental conditions, the M and E oscillators drive the

82   morning and evening anticipatory increments of locomotor activity, respectively. The M

83   oscillator is also the master pacemaker of the free-running locomotor rhythms in constant

84   darkness (DD) (8–10).

85   Neuropeptide PDF as well as the unique composition and regulatory mechanisms of the

86   molecular clock underlie the distinct role of the M oscillator. The main negative-feedback loop

87   of the M oscillator's molecular clock employs a specific phosphorylation program that regulates

88   the nuclear translocation of PER/TIM complex (11). The nuclear receptor UNFULFILLED

89   (UNF) is almost uniquely present in the lateral neurons within the circadian circuit (12,13). UNF

90   accumulates rhythmically in the s-LNvs and, in cooperation with another nuclear receptor E75,

91   enhances CLK-dependent *per* transcription. Thus, UNF and E75 consist a positive limb of an

92   additional feedback loop in specific to the s-LNv molecular clock. Because UNF and E75 also

93   play critical roles in the development of the s-LNvs, knockdown of either gene during

94   development or adulthood results in low rhythmicity and extended period, respectively (12,14).

95   Nuclear receptors (NRs) are a superfamily of proteins that function as ligand-dependent

96   transcriptional regulators (15). The ligands are small lipophilic molecules that can diffuse across

97   the cell membrane, such as thyroid and steroid hormones. In *Drosophila melanogaster*, only two

98   lipophilic hormones, 20-hydroxyecdyson (20E) and the sesquiterpenoid juvenile hormone (JH)

99   are known nuclear receptor ligands, which have critical roles in developmental processes,

100   including molting, puparium formation, and neurogenesis (15–17). Although many NRs remain

101   orphan without a known ligand, diatomic gases nitric oxide (NO) and carbon monoxide (CO)

102   can bind and regulate the activity of some NRs. Several studies have demonstrated *in vitro* and

103   *in vivo* that NO binds to E75 and regulates its interaction with DHR3 (18), SMRTER (19), and

104   UNF (20) in different tissues during development. Thus, the binding of NO to E75 confers an

105   important switching mechanism in various developmental processes.

106   NO is an unconventional messenger involved in numerous biological functions, including

107   immune defense, respiration, intracellular signaling and neurotransmission (21,22). NO can act

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108 locally near the source of its production. It can diffuse across membranes and also act as a long-

109 range signaling molecule (22,23). NO signaling is broadly classified into the classical pathway

110 mediated by cGMP and cGMP-independent non-classical one involving diverse mechanisms

111 such as posttranslational modifications and transcriptional regulations (22,24). In mammals, the

112 importance of NO signaling in the light-dependent phase-resetting and maintenance of

113 rhythmicity (25–28) is established. These effects were largely explained by the canonical

114 NO/cGMP signaling (29–31). However, whether NO has a regulatory role in *Drosophila*

115 circadian behavior has never been addressed.

116 Here we explore the role of NO in circadian locomotor behavior of *Drosophila* using

117 multiple genetic approaches. We present evidence that NO signaling is necessary for both light-

118 dependent and free-running circadian behavior. NO acts cell-autonomously as well as non-cell-

119 autonomously at multiple processes required for generating behavior, including axonal

120 morphogenesis, pacing of molecular clocks and output control. We identify glial cells as a major

121 source of NO that controls free-running locomotor output. Our results highlight the complexity

122 of locomotor behavior regulation and oft-neglected importance of glia in the regulation of

123 behavior.

Results

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126 ***dNOS* deletion mutants show abnormal circadian behavior**

127 NO is chiefly produced by an enzyme nitric oxide synthase (NOS) through the conversion of

128 arginine into citrulline using NADPH as a cofactor (32,33). Three distinct NOS isoforms

129 (endothelial e-NOS, inducible i-NOS, and neuronal n-NOS) exist in mammals, whereas

130 *Drosophila* has a single *NOS* (*dNOS*) gene that produces 10 splice variants (Fig. S1) (34). Since

131 NOS functions as homodimers, alternatively spliced variants, most of which encode truncated

132 proteins, are proposed to act as dominant negatives (35). To investigate the possible roles of NO

133 in fly circadian rhythms, we took advantage of two *NOS* CRISPR deletion mutants, *NOS Δall,*

134 and *NOS Δter* (20). The former has a deletion of the entire NOS locus, while the latter is a

135   partial deletion mutant lacking exons 1 to 6 but bears intact two uncharacterized genes within the

136   *NOS* locus (Fig 1A). RT-qPCR using the primers targeting the exons commonly included in all

137   variants (exon 10 and 11) confirmed the absence of the full-length NOS1 mRNA expression in

138   *NOS Δall* mutants (Fig. 1B) A reduced level of the product was detected in *NOS Δter* mutants,

139   consistent with the location of the deletion. Furthermore, we directly measured NO production in

140   cultured whole brains using a fluorescent dye DAR4-M (36). NO was virtually undetectable in

141   both *NOS Δall* and *NOS Δter* strains in this assay, confirming that both are complete loss-of-

142   function mutants (Fig. 1C).

143   Having validated the deletion mutants, we next tested their locomotor activities in LD

144   and DD paradigms. Homozygous mutants had strongly reduced rhythmicity in DD. Trans-

145   heterozygous of two deletion alleles was equally detrimental to DD rhythmicity, whereas

146   heterozygous mutations had no effect on rhythmicity (Table 1 and Fig. 2A). Moreover, morning

147   activity patterns in LD were strongly impaired in homozygous and trans-heterozygous mutants,

148   nearly lacking both anticipatory increments of activity before lights-on and the startling reaction

149   to light (Fig. 2B). The severe reduction of the startling response to light suggests an impairment

150   in photoreception through the compound eyes known to be necessary for this phenomenon

151   (37,38). This is consistent with the fact that NO/cGMP signaling is necessary for neurites

152   patterning of the receptor neurons in the optic lobe during development (39). On the other hand,

153   low rhythmicity in DD and poor morning anticipation are indicative of the dysfunction of the s-

154   LNvs, the Master and Morning oscillator.

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156   **NOS is a regulator of morphogenesis of the s-LNv axons**

157   NO signaling plays critical roles in various developmental processes in the nervous system,

158   including neurite patterning of the visual system and axon pruning/regrowth of mushroom body

159   (MB) neurons (18–20,39,40). Therefore, to explore the possible effect of *NOS* loss-of-function

160   on the development of the s-LNvs, we expressed a membrane-targeted yellow fluorescent

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161   protein mCD8::VENUS with the *gal1118-Gal4* driver and visually inspected s-LNv axonal

162   morphology. To our surprise, normally a very orderly branching pattern of terminal neurites was

163   severely disturbed in *NOS* homozygous mutants (Fig. 3A). The neurites were extended in length

164   and branching pattern was highly disordered and fuzzy (Fig. 3A and B). Circadian change of

165   axonal termini (41) was not evident in mutants due to the highly disordered structure. This

166   disorderly morphology is reminiscent of the axon pruning defects of MB neurons in *NOS*

167   deletion mutants (20). Since NO promotes MB neuron axon pruning and degeneration by

168   inhibiting UNF/E75 heterodimer formation (20), we wondered if similar mechanisms are

169   involved in the structural maturation of the s-LNvs. To probe this idea, we visualized s-LNv

170   projections by expressing mCD8::VENUS while constitutively knocking down UNF. We found

171   that s-LNv axonal morphology was affected in a similar manner in this condition, having more

172   numerous and disordered neurites than the control (Suppl. Fig S2). These results suggest that

173   NOS is necessary for axonal morphogenesis of the s-LNvs and part of its role may be via

174   controlling the activity of UNF.

175   To better understand the nature of the low rhythmicity in *NOS* mutants, we also

176   performed around-the-clock immunostaining of a key clock component PER on the third day of

177   constant darkness (DD3). Neither the phase nor the amplitude of the molecular clocks of the s-

178   LNvs was affected in *NOS* mutants. Molecular clocks of the LNds also maintained high-

179   amplitude 24-h rhythms in mutants (Fig. 3C). Therefore, the arrhythmic behavioral phenotype of

180   *NOS Δ* mutants is uncoupled from the state of the molecular clocks and caused by the

181   developmental impairments. It probably entails the wrong synaptic connectivity of the

182   malformed s-LNvs axonal terminals, in addition to defects in possibly many other cells involved

183   in locomotor output. Malformation of s-LNv axons is likely to be also responsible for lack of

184   morning anticipatory behavior.

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186   **NO from diverse cellular groups can modulate the state of the molecular clocks and**

187   **behavioral output**

188   Whereas NOS is undoubtedly important for developmental processes, the fact that NO continues

189   to be produced in the brains of adult flies (Fig. 1C) suggests that NO may also have a post-

190   developmental role in regulating circadian rhythms. It was previously shown using an anti-NOS

191   serum (42) that NOS is expressed almost everywhere in the brain. However, the anti-NOS serum

192   does not distinguish various NOS isoforms and thus the picture may not be identical to the loci

193   of active NO production. Separately, classical histochemical studies of NADPH diaphorase

194   activity of NOS and soluble guanylate cyclase (sGC)/cGMP immunohistochemistry have

195   suggested that NOS is active in sensory pathways including visual system, in memory circuits

196   including the calyx of mushroom body, in the central complex and also in some glial cells

197   (32,39,43–45). Since these were all indirect assessments of NO production, we analyzed the

198   localization of NO in the brain using the NO-specific fluorescent probe DAR4-M. DAR4-M

199   staining showed distinct patterns of cell bodies and neurites in many areas. The signal was

200   particularly high within and around the central complex and in the optic lobe, with cell bodies

201   arranged in concentric semicircles reminiscent of the laminar and medulla glial cells (46) (Figure

202   4A). These patterns were overall similar to those described for the localization of NADPH

203   diaphorase activity and cGC/cGMP. In addition, since DAR4-M staining is not sensitive enough

204   to assess daily variation of NO production, we examined the temporal expression pattern of the

205   functional isoform *dNOS1* using qPCR. We found that mRNA of the *dNOS1* was rhythmically

206   expressed in the fly head, peaking around ZT10 in LD (Fig. 4B), suggesting that overall NO

207   levels in the brain may be circadian.

208   Interestingly, we detected a slight enrichment of NO-positive fluorescence within the s-

209   LNvs marked with *gal1118 > mCD8::Venus* (Fig. 4C). This was unexpected because previous

210   transcriptome studies found very little or no *NOS* expression within the s-LNvs (13,47,48). This

211   suggests that NO produced elsewhere migrates to the s-LNvs. Within the s-LNvs, transcriptional

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212   regulation by E75 and UNF is a unique and important node of the molecular clockwork (12,14).

213   Intriguingly, it was shown that heterodimerization of E75 and UNF is controlled by NO *in vivo*

214   and *in vitro* (20). Therefore, we asked whether the state of the molecular clocks can be

215   modulated by increasing NO within the s-LNvs.

216   To this end, we overexpressed a macrophage-derived constitutively active NOS

217   (macNOS) under the UAS control (18) using *Pdf-Gal4* and performed PER staining on DD3

218   every 4 h (Fig. 4D). The increase of NO within the PDF positive neurons was confirmed by

219   DAR4-M staining (Suppl. Fig. S3). This manipulation lead to a delay of the PER induction phase

220   by about 4 h without dampening the amplitude of PER rhythms. The results are consistent with

221   the known role of UNF and E75 and regulation of their heterodimerization with NO: high levels

222   of NO disrupt UNF/E75 dimer and delays PER rising phase that is normally enhanced by

223   UNF/E75.

224   *Pdf>macNOS* flies had a slight extension of free-running period and the reduction in

225   rhythmicity. But the reduced rhythmicity was not statistically significant compared with the

226   driver control. Because *Pdf-GAL4* is expressed in both s- and l-LNvs, these phenotypes may be a

227   compound effect from both cell types. When we expressed macNOS under the s-LNv-specific

228   *R6-Gal4* driver, we observed a reduction in rhythmicity but no differences in the period length

229   (Table 1). Neither manipulation consistently affected behavioral patterns in LD (Suppl. Fig.S4).

230   Therefore, the major behavioral consequence of forced NO production in the s-LNvs is a

231   reduction in free-running rhythmicity. This is probably a consequence of the misalignment of

232   molecular phases between the s-LNvs and other clock neurons (Fig. 4D).

233   The results of the DAR4-M staining and the finding that NO can regulate the state of the

234   molecular clocks prompted us to investigate whether NO produced in specific cell types or brain

235   area is important for normal circadian locomotor activity. Therefore, taking into account the

236   notion that NO can act both locally and remotely, we selected a set of GAL4 drivers and drove

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237   the expression of macNOS. Locomotor activity of these flies was assayed in standard LD-DD

238   conditions.

239   As summarized in Table 1, we used two clock cell-specific drivers *Tim-GAL4* and

240   *Clk1982-GAL4*; a mushroom body-specific driver *DH52-GAL4*; three generic optic lobe-specific

241   drivers *GMR33H10-GAL4*, *GMR79D04GAL4-*, and *GMR85B12-GAL4 (Suppl. Table S1)*; a glia-

242   specific driver *Repo-GAL4*; and two pan-neuronal drivers *elav-GAL4* and *R57C10-GAL4*.

243   Strikingly, all of them except *elav-GAL4* induced a reduction of rhythmicity upon

244   overexpression of macNOS. This is most likely because pan-neuronal *elav-GAL4* is a weaker

245   driver than another pan-neuronal driver *R57C10-GAL4* .The reduction of rhythmicity was

246   markedly dramatic with *Repo*, *tim* and optic lobe specific drivers. Collectively, these results

247   indicate that the overproduction of NO is generally disruptive to locomotor rhythms and suggest

248   that the NO production and clearance should be tightly regulated. LD behavior was not

249   obviously affected in any manipulation (Suppl. Fig. S4).

250   To find cell types that natively produce and secrete NO and contribute to the control of

251   locomotor rhythms, we next performed an opposite experiment. We expressed RNAi against

252   NOS (VDRC #27725) using a similar set of drivers and analyzed its effects on behavioral

253   rhythms (Table 1). Consistent with the likely absence of NOS within the s-LNvs, NOS RNAi

254   with *Pdf-GAL4* and *R6-GAL4* did not show any behavioral phenotype. NOS RNAi driven with a

255   mushroom body driver *DH52-GAL4* caused a reduced rhythmicity, whereas *macNOS* expression

256   with the same driver had no effect. Most of the other drivers that disrupted rhythms with

257   *macNOS* expression also reduced behavioral rhythmicity with *NOS* RNAi. These include a pan-

258   neuronal driver *R57C10-Gal4*, optic-lobe drivers *79D040-Gal4* and *85B12-Gal4*.The strongest

259   effect was observed with *Repo-GAL4* and *tim-Gal4*, whereas there was no effect with *Clk1982-*

260   *Gal4*. Since the expression of *Clk1982-Gal4* is relatively restricted to CLK-positive neurons,

261   these results suggest TIM-positive glial cells as an important source of NO in the regulation of

262   circadian locomotion. *Repo-Gal4* driving a second independent RNAi against NOS (TRiP

263 #50675) also reduced free-running rhythmicity (3% rhythmic, period 24.0 ± 0 h, N=31). Another

264 VDRC RNAi line (#108433) had no effect on behavior with any driver, which is most likely due

265 to an inefficient knockdown compared to the VDRC #27725 line, judging from the NO staining

266 intensity (Supplementary figure Fig S5). Behavioral patterns in LD was not affected by NOS

267 knockdown in any driver (Suppl. Fig. S4). Altogether, the results of NOS gain- and loss-of-

268 function mini screens indicate that NO produced in many different cell types excluding

269 pacemaker neurons contribute to generate normal free-running locomotor rhythms.

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271 **NO produced in glia plays an active role in the regulation of locomotor output**

272 Constitutive NOS knockdown may induce developmental malformations in the brain that lead to

273 the reduction of rhythmicity, as evidenced by the phenotypes of *NOS Δ* mutants (Figs. 2A, 2B,

274 3A and 3B). Therefore, to test if NOS is required for active maintenance of rhythmicity after

275 eclosion, we performed the adult-specific knockdown of NOS using pan-neuronal, optic lobe

276 specific and glial GAL4 drivers combined with the temperature-sensitive GAL4 repressor,

277 GAL80^{ts} (49) (Table 1). Glia-specific NOS knockdown caused a notably strong reduction of

278 rhythmicity. In addition, NOS RNAi driven by the pan-neuronal *R57C10-Gal4* extended the

279 free-running period. These results indicate an indispensable role of NO produced in glia for

280 generating circadian locomotor output in adult flies, as well as an existence of a neuronal circuit

281 through which NO signaling regulates the free-running period.

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Discussion

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285 Gaseous signaling molecules play important roles in a myriad of biological processes, including

286 circadian rhythms in mammals. NO and CO were proposed to be "light" and "dark"- induced

287 messengers that convey information from zeitgebers to the core of the molecular clocks

288 (26,50,51). Here we investigated the possible involvement of NO in *Drosophila* circadian

289   rhythms. Our results overall suggests that NO exerts a coordinated, temporarily and spatially

290   diverse effect on the *Drosophila* circadian system.

291   It is rather surprising that the lack of NOS enzyme is not lethal as NO is part of various

292   developmental processes (18–20,39,52,53). Nonetheless, *NOS Δ* mutants are strongly

293   arrhythmic, incapable of morning anticipation and exhibit minimum light-induced startle

294   response. Whereas the absence of light-induced startle phenotype is likely to be explained by

295   disruptions in light reception through wrong patterning of the receptor cells of the compound eye

296   (39), lack of anticipation point to the problems with the morning pacemakers, the s-LNvs.

297   Indeed, axonal terminals of these neurons in the mutants have an utterly wrong shape, suggesting

298   the wrong or absent synaptic connections with the downstream partners. Together with the

299   axonal morphology phenotype induced by *UNF* knockdown, it is plausible that NO acts through

300   *UNF* and its dimerization partner E75 to define the state of the axonal terminals during

301   development. As in the case of MB neurons (20), this process might involve axon pruning and

302   regrowth.

303   The functional isoform *dNOS1* showed a circadian variation of its RNA levels throughout

304   the day, which suggest that levels of NO could cycle. However, *dNOS* is likely to be regulated

305   by its truncated isoforms in a stage- and cell-type-specific manner, which lays an additional

306   complexity to the regulation of NO production and probably leads to the heterogeneous and

307   context-specific variations of NO. Whether its cycling is important or not for the rhythmicity, it

308   is clear that NO has only a modulatory role in the s-LNvs' molecular clocks, since UNF or E75

309   knockdown in the s-LNvs have much stronger effect on molecular clockwork, i.e. dampening of

310   the PER cycling amplitude and period extension (14,12).

311   In our *NOS-RNAi* mini screen, two non-clock neurons-specific drivers, *GMR79D04* and

312   *GMR85B12* reduced locomotor rhythmicity in DD. This phenotype was rather specific to

313   developmental stage, reinforcing the idea that NO is necessary for a proper establishment of

314   neuronal circuits. A low rhythmicity phenotype produced by knockdown under pan-neuronal

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315   driver *GMR57C10-GAL4* backs up this idea. Intriguingly, however, in addition to the low

316   rhythmicity, *GMR57C10 > NOS RNAi* in adulthood resulted in an extended period. This raises a

317   question on what might be the neuronal subsets that produce NO and contribute to the regulation

318   of period length of locomotor activity. The effect must be of a different nature from the

319   regulation of *per* transcription by UNF/E75. An interesting hint comes from a recent study by the

320   group of G. Rubin (54), which shows that NO acts as a co-transmitter in a subset of

321   dopaminergic neurons, specifically in some of the PAMs, PPL1s and PPL2abs. It is thus possible

322   that dopamine signaling regulated by NO is involved in the control of locomotor activity period.

323   Targeting glial cells leads to the strongest and most persistent phenotype in locomotor

324   activity both for gain- and loss-of-function of NOS. The importance of glia in circadian rhythms

325   have been recognized, especially those expressing the molecular clocks and exert reciprocal

326   communication with the pacemaker neural circuit (55,56). Our study is the first to identify NO as

327   a signaling molecule produced in glia that mediates part of the role of glia in circadian

328   behavioral in flies. It has been shown that in mammals NO mediates light-induced phase-shifts

329   through the cGMP pathway (31). It is an interesting parallel to note that forced production of NO

330   in the s-LNvs caused phase shift rather than amplitude dampening, although we speculate that

331   part of this effect comes from NO hampering E75/UNF dimerization that normally enhances

332   CLK/CYC-mediated *per* transcription. Mammalian clocks contain E75 homologs REV-ERB α/β

333   that work with another nuclear receptor, ROR. In contrast to flies, REV-ERB α/β have a

334   repressive function within the clocks, inhibiting transcription of *BMAL1*, a mammalian analog of

335   *CYC*. Interestingly, *in vitro* studies of mammalian cell culture showed that excessive presence of

336   NO increases the production of *BMAL1*, consistently with the hypothesis that NO decreases

337   REV-ERB α/β activity (57). These findings altogether point out that NO is an evolutionarily

338   conserved regulator of circadian rhythms.

339   In line with recent studies (7,58,59), our research expands the view on the factors that

340   participate in neuronal and molecular mechanisms of circadian rhythmicity. The finding that

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341   gaseous messenger NO contributes to the various aspects of circadian rhythmicity, from

342   development to the maintenance, emphasizes that non-cell-autonomous, systemic regulation is

343   integral to the circadian circuit operation. Our results set a foundation for future studies

344   addressing whether or not specific glial or neuronal classes are required in this regulation and

345   how the NO signaling modulates the state of the pacemaker circuit.

Materials and Methods

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Fly rearing, crosses, and strains

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349   *Drosophila* were reared at 25 °C on a corn-meal medium under 12 hr:12 hr light-dark (LD)

350   cycles. Two CRISPR deletion mutants *NOS Δter*, *NOS Δall* were kindly provided by O.

351   Schuldiner (20). The *UAS-macNOS* line was originally generated by H. Krause (18) and

352   provided also by O. Schuldiner. The drivers *GMR57C10*, *GMR79D04*, *GMR85B12*, *GMR33H10*

353   (60) as well as *UAS-NOS-RNAi[56675]* were obtained from Bloomington Stock Center (Indiana,

354   US). The UAS lines *NOS-RNAi[27725]* and *NOS-RNAi[108433]* were obtained from the Vienna

355   *Drosophila* Resource Centre (VDRC). The *Clk1982-Gal4* line was provided by N.R. Glossop

356   (61). The lines *Pdf-Gal4* (62), *Repo-Gal4* (63), *OK107-Gal4* (64), *D52H-Gal4* (65) , *GMR-Gal4*

357   (66), *Elav-Gal4* (67), *R6-Gal4* (68), *UAS-miR unf* (12,14) were described previously.

Behavioral Assays

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360   The locomotor behavior assay was performed as described previously (12) and data were

361   analyzed using FaasX software (69). Briefly, male flies were first entrained in 12 h/12 h LD

362   cycles for 4 days and then released in DD for 7–10 days. The flies with power over 20 and width

363   over 2.5h according to the χ^2 periodogram analysis were defined as rhythmic. The significance

364   threshold was set to 5%. The χ^2 test was used to compare the rhythmicity of the flies, and the

365   Student's *t* test (2-tailed) was used to compare the free-running period.

Immunocytochemistry, microscopy and quantification

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368   The brains were imaged using a Leica SP5 confocal microscope. At least 10 brain hemispheres

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369   were subjected to analysis using Image J software (National Institutes of Health). The anti-PER

370   signal was quantified as previously described (12).

371   ***Nitric oxide visualization and measurements***
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373   NO visualization was performed as described in (20) with minor modifications. Brains were

374   dissected in PBS and incubated with 10 μ M Diaminorhodamine-4M AM (DAR-4M, Sigma-

375   Aldrich) in PBS for 1 h at RT, followed by the fixation for 15 min in PBS containing 4%

376   paraformaldehyde. Immediately after the fixation brains were mounted and imaged. For NO

377   measurements at different times of the day, the procedure was exactly the same with the

378   omission of the fixation step. Long-term NO measurement in *ex-vivo* brain culture was

379   performed as described in (70). Briefly, brains were dissected on an ice-cold plate in modified

380   Schneider's medium (SM$^{\text{active}}$) (71) with an addition of 5 mM Bis-Tris (Sigma) and then

381   mounted on a glass-bottom dish (35 mm MatTek petri dish, 20 mm microwell with 0.16/0.19

382   mm coverglass). The glass-bottom well was filled with the SM$^{\text{active}}$ medium with 10 μ M

383   DAR-4M. Time-lapse imaging was performed at 25 $^\circ$ C and 80%, with images acquired every

384   hour.

385   ***RNA analysis***
386
387   Total RNA was isolated from adult fly heads using Trizol (Invitrogen) following the

388   manufacturer's protocol. The RNA was reverse-transcribed using oligo(dT) primers, and the

389   resulting cDNAs were quantified using real-time qPCR as previously described (47). The mRNA

390   levels of *dNOS1* were normalized to those of *elongation factor 1 (Ef1).*

Acknowledgments

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588
589

590   ## Figure Legends
591
592   **Figure 1. *NOS Δ* mutants do not produce nitric oxide.** (A) *NOS* gene, *NOS Δ* all, and *NOS Δ*

593   *ter* mutants and two reverse genes residing within the *NOS* locus. (B) mRNA levels of *NOS1*, a

594   full-length functional isoform of NOS (NOSd1), at ZT2 in the heads of *NOS Δ* mutants and w^{1118}

595 were analyzed using qPCR.**$p<0.01$ (Student's test). (C) NO levels were measured using

596 DAR4-M dye in the brains of *NOS Δ* mutants and w^{1118} in brain explants for 4 h using timelapse

597 microscopy. **$p<0.01$ (Student's test)

598

599 **Figure 2. *NOS* deletion impairs the circadian behavior in light-dark cycles and constant**

600 **darkness.** (A) Locomotor activity histograms for group average activity of w^{1118} and *NOS Δ*

601 mutants. (N=32)(B) Histograms of group average activities of w^{1118} and *NOS Δ* mutants in LD,

602 an average of 3 days.

603

604 **Figure 3. *NOS* deletion causes the malformation of the axons of the s-LNvs but does not**

605 **affect their molecular clocks.** (A) s-LNv axonal terminal projections were visualized by

606 expressing *mCD8::Venus* and staining with anti-GFP antibodies at ZT2 and ZT14.

607 Representative confocal images are shown. (B) Quantifications of the length of the terminal

608 branches at ZT2**.** (C) PER levels of w^{1118} and *NOS Δ* mutants in the s-LNvs and LNds analyzed

609 every 4 h on DD3. NOS deletion does not affect PER rhythms.

610

611 **Figure 4. NO production in the brain and its effect on the s-LNv molecular clocks.** (A)

612 Intracellular NO staining with DAR4-M staining showed an accumulation of NO in the optic

613 lobe (OL) and in and around the central complex (CCX) of w^{1118} flies. (B) Around-the-clock

614 measurement of the *dNOS1* isoform mRNA levels by qPCR in LD. *$p<0.05$, **$p<0.01$,

615 ***$p<0.001$ (one-way ANOVA test) (C) Enrichment of the DAR4-M signal within the s-LNv

616 cell body. (D) PER levels of w^{1118} and *PDF>macNOS* mutants were measured every 4 h on

617 DD3. Red and black arrows point at the trough of PER rhythms in *Pdf > macNOS* and control

618 flies, respectively. PER rhythms are delayed by approximately 4 hours in *PDF>macNOS* flies.

619 **Supporting Information Captions**

620

621 **Supplementary Figure 1. NOS splice isoforms.** dNOS1 and presumably dNOS8 encode

622 functional complete enzyme. The rest isoforms lead to a truncated enzyme. Information is taken

623 from (34). dNOS1-specific primers used are (F) GGC GAG CTT TTC TCC CAG GA, and (R)

624 GAC GAG CCA ATG CTG GAG TC, indicated in red.

625

626 **Supplementary Figure 2. Axonal morphogenesis of the s-LNvs relies on UNF.** s-LNv axonal

627 terminals in control (*Pdf-GAL4*/+) and flies with LNv-targeted UNF knockdown (*Pdf-GAL4*

628 driving *UAS-miR unf*, shown as *PDF> UNF-RNAi*) were visualized by co-expressing

629 *mCD8::Venus*. Flies were dissected at ZT2 and stained with anti-GFP antibodies. Representative

630 confocal images are shown.

631

632 **Supplementary Figure 3. Upregulation of NO upon macNOS overexpression.** DAR4-M

633 staining of brain expressing *macNOS* in *Pdf-Gal4*. Left, Representative confocal images. Right,

634 comparison of DAR4-M fluorescent levels of a single representative WT and *Pdf > macNOS*

635 brains within the region of s-LNvs.

636

637 **Supplementary Figure 4. Cell-restricted manipulation of NOS does not affect LD**

638 **locomotor behavior.** Locomotor activity histograms for group average activity of macNOS or

639 NOS-RNAi[27725] expressed under indicated drivers. 4 days of LD are shown.

640

641 **Supplementary Figure 5 NOS-RNAi efficiency comparison.** DAR4-M staining measures of

642 two *Elav*-driven NOS-RNAi[27725] and NOS-RNAi[108433]. Fluorescence levels were measured

643 broadly in the region of the central brain, approximately in the area of the central complex.

644 RNAi line 27725 induces a significant reduction of DAR4-M signal. **p<0.01 (Student's test).

645 ## Tables
646
647 **Table 1. Free-running locomotor rhythms.**

648
649

Temperature	Genotype	Period ± SEM (hr)	Power ± SEM	n	%R
25°C	*W1118*	23.5±0.05	167.5±9.3	124	93.6
	CantonS	24.1±0.35	102.3±18.0	27	84.4
	NOSter/+	23.7±0.04	217.7±10.5	127	91.3
	NOSter	23.2±0.2	84.3±29.0	105	21.0***
	NOSall/+	23.7±0.04	245.1±12.1	126	76.9
	NOSall	23.6±0.06	149.4±15.5	173	56.1**
	NOSall/NOSter	23.5±0.2	79.0±17.4	59	66.1
macNOS oxp	*>macNOS*	23.4±0.05	59.4±2.1	89	85.4
LNvs	*PDF>macNOS*	24.5±0.15***	51.7±6.1	25	76.6
s-LNVs	*R6>macNOS*	23.8±0.09	69.9±5.2	31	65.5**
MB	*D52H>macNOS*	24.2±0.05	145.2±13.9	28	92.9
Clock neurons	*tim>macNOS*	24.7±0.1****	61.3±6.3	30	36.7****
Clock neurons	*Clk1982>macNOS*	23.9±0.05	85.5±7.6	30	76.7
OL	*GMR33H10>macNOS*	23.7±0.09	85.2±8.7	62	53.2****
OL	*GMR79D04>macNOS*	23.9±0.09	43.3±4.9	63	28.6****
OL	*GMR85B12>macNOS*	23.6±0.1	53.1±7.9	60	61.7***
Glia	*Repo>macNOS*	23.5±0.05	98.2±5.0	28	29.8****
Pan-neuronal	*GMR57C10>macNOS*	25.0±0.4*	65.9±12.9	61	32.8****
Pan-neuronal	*Elav>macNOS*	23.7±0.05	83.5±6.4	59	81.4
NOS KD	*NOS-RNAi^{27725}/+*	23.7±0.04	110.7±0.04	145	84.0
LNvs	*PDF>NOS-RNAi*	24.4±0.03	184.1±13.9	26	100
s-LNVs	*R6>NOS-RNAi*	23.6±0.04	113.0±5.5	60	80
MB	*D52H>NOS-RNAi*	23.6±0.4	105.4±10.5	29	65.5*
MB	*OK107>NOS-RNAi*	23.5±0.05	141.2±12.0	32	96.9
Photoreceptors	*GMR>NOS-RNAi*	23.6±0.06	200.1±12.3	60	95.2
Clock neurons	*tim>NOS-RNAi*	24.4±0.2	166.2±10.4	47	38.3****
Clock neurons	*Clk1982>NOS-RNAi*	23.6±0.02	173.3±5.03	62	88.7
OL	*GMR33H10>NOS-RNAi*	23.5±0.03	164±7.1	32	96.9
OL	*GMR79D04 >NOS-RNAi*	25.1±0.2**	105.2±7.9	59	61.0**
OL	*GMR85B12 >NOS-RNAi*	23.6±0.03	121.3±6.9	44	70.5**
Glia	*Repo>NOS-RNAi*	23.6±0.2	79.4±6.6	63	32.1****
Pan-neuronal	*GMR57C10>NOS-RNAi*	26.2±0.2****	121.2±8.2	60	55.0**
Pan-neuronal	*elav>NOS-RNAi*	23.5±0.05	122.3±9.1	95	76.8
CTR	*PDF>*	24.1±0.04	132.7±6.9	58	96.8
	R6>	23.4±0.05	105.2±5.7	45	85.1
	D52H>	23.9±0.04	177.3±8.3	10	90.5
	OK107>	23.7±0.06	139.6±8.5	30	96.8
	GMR	23.6±0.03	187.5±6.1	28	93.3
	Tim>	24.1±0.05	125.4±6.3	82	90.2
	Clk1982>	23.6±0.03	150.6±7.4	60	86.7

22

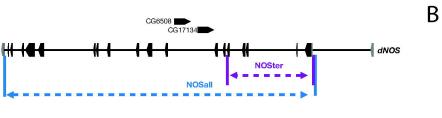|					
	GMR33H10>	23.1±0.05	144.9±6.2	60	85.0
	GMR79D04 >	24.3±0.2	96.6±5.5	64	84.4
	GMR85B12 >	23.5±0.04	128.5±6.9	91	89.0
	Repo>	23.3±0.05	109.2±6.9	83	82.0
	GMR57C10>	24.2±0.2	117.9±4.9	60	78.3
	Elav>	23.7±0.05	143.0±7.8	120	82.8
18°C ->29°C Adult-only KD	Repo>NOS-RNAi	24.1±0.5	47.2±7.3	21	47.6*
	GMR79D04>NOS-RNAi	24.7±0.3	93.1±8.3	28	75.0
	GMR85B12>NOS-RNAi	25.6±0.3	86.0±12.5	17	56.7
	GMR57C10>NOS-RNAi	26.8±0.07***	165.3±11.5	25	96.0
CTR	NOS-RNAi/Tub-Gal80ts	25.6±0.3	89.2±6.5	29	79.3
	Repo>	23.5±0.6	114.2±9.3	29	72.4
	GMR79D04>	23.3±0.07	76.0±8.7	28	67.9
	GMR85B12>	23.2±0.09	74.2±15.1	28	32.1
	GMR57C10>	23.2±0.06	121.7±9.74	30	86.7

650

651 ^{a}n, Number of flies; %R, % of rhythmic flies. Rhythmicity was compared with the driver control

652 or with control genotypes (*CantonS* for *NOS* mutants) by a χ^2 test. Periods were compared

653 with the controls (Student's *t* test): *p 0.05; **p 0.01; ***p 0.001; ****p 0.0001.
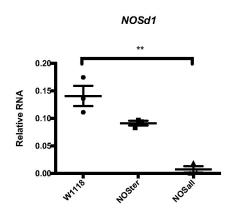
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23

657 **Supplementary Table S1. Optic lobe-specific drivers**.
658 Distribution and intensity of the tested generic optic lobe-specific drivers, taken from
659 Janelia Fly Light project. Original characterization was based on the GFP expression. All
660 three optic lobe-specific drivers are enriched in the optic lobes. Occasional expression
661 outside OL is mainly relatively weak in compare with expression in OL.
662

	R33H10	R79D04	R85B12	R57C10
antennal lobe	1_2	1_1	1_5	
antennal mechanosensory and motor center	1_3	0_5	5,0	
anterior ventrolateral protocerebrum	3,0	2_1	1_1	
antler	1_1	1,0	0_5	
bulb	1_4	1,0	1,0	
cantle	1_2	0_5	1,0	
crepine	1_1	0_5	1_1	
ellipsoid body	0_5	0_5	0_5	
epaulette	1_4	2_2	1_1	
fan-shaped body	1_1	2,0	1,0	
flange	2_1	2_1	2_2	
gall	1_2	0_5	0_5	
gorget	1_2	3_1	1_1	
inferior bridge	1_1	1,0	1,0	
inferior clamp	1_2	1_1	1_1	
inferior posterior slope	1_2	1,0	1_1	
lateral accessory lobe	1_2	3,0	1_1	
lateral horn	1_1	1_2	1,0	
mushroom body	1_1	1,0	1_2	
noduli	0_5	0_5	0_5	
optic lobe	5_2	5_2	5_2	
optic tubercle	2,0	0_5	2,0	
posterior lateral protocerebrum	1_2	2_1	1,0	
posterior ventrolateral protocerebrum	2_1	1_3	1_2	
protocerebral bridge	0_5	1,0	1,0	
prow	1_4	2_3	4_1	
saddle	2_1	1,0	4,0	
subesophageal ganglion	3_2	3,0	2_1	
superior clamp	2_1	1_1	1,0	
superior intermediate protocerebrum	2_1	1,0	1_1	
superior lateral protocerebrum	1_2	1,0	2,0	
superior medial protocerebrum	2_1	1_1	1_2	
superior posterior slope	1_1	1_1	0_5	
vesta	1_4	0_5	1,0	
Gene region		Src64b	CG9650	zf30c	Nsyb
Intensity			**Distribution**		

Value	Description	Criteria for selection		Value	Description	Criteria for selection
0	blank	No pattern		0	passing tract	Tracts passing neuropil
1	faint	Visible with enhanced contrast only		1	sparse	Like one glom in al
2	very weak	Visible when known with nc82 staining		2	local, regional	Bigger region or multiple glom in al
3	weak	Easily visible with nc82 staining		3	widespread	Less than half of the structure
4	strong	Bright		4	prevalent	More than half of the structure
5	very strong	Really bright		5	ubiquitous	Everywhere

663

664

665

Figure 1

Figure 2

A

B

Figure 3

Figure 4

A

DAR4M

DAR4M mCD8::GFP

Me

Lo

CCX

B

Relative RNA

ZT

C

DAR4M

PDF>mCD8::GFP

D

PDF PER

>macNOS

PDF>macNOS

sLNVs, PER

>macNOS
PDF>macNOS

CT, DD3