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A Conserved Circadian Function for the Neurofibromatosis 1 Gene

Graphical Abstract

Highlights

- Loss of Nf1 affects calcium levels in a circadian circuit in Drosophila
- Nf1 interacts with peptidergic signaling to influence behavior
- Mammalian astrocytes lacking Nf1 show dampened cycling of calcium

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In Brief
Bai et al. show that the gene mutated in the disease Neurofibromatosis 1 is required for maintaining levels or cycling of calcium in circadian neurons in Drosophila and in mammalian cells. These effects likely account for effects of Nf1 on circadian behavior in Drosophila and may be relevant in explaining sleep phenotypes in patients.

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A Conserved Circadian Function for the Neurofibromatosis 1 Gene

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SUMMARY

Loss of the Neurofibromatosis 1 (Nf1) protein, neurofibromin, in Drosophila disrupts circadian rhythms of locomotor activity without impairing central clock function, suggesting effects downstream of the clock. However, the relevant cellular mechanisms are not known. Leveraging the discovery of output circuits for locomotor rhythms, we dissected cellular actions of neurofibromin in recently identified substrates. Herein, we show that neurofibromin affects the levels and cycling of calcium in multiple circadian peptidergic neurons. A prominent site of action is the pars intercerebralis (PI), the fly equivalent of the hypothalamus, with cell-autonomous effects of Nf1 in PI cells that secrete DH44. Nf1 interacts genetically with peptide signaling to affect circadian behavior. We extended these studies to mammals to demonstrate that mouse astrocytes exhibit a 24-hr rhythm of calcium levels, which is also attenuated by lack of neurofibromin. These findings establish a conserved role for neurofibromin in intracellular signaling rhythms within the nervous system.

INTRODUCTION

The Neurofibromatosis 1 (Nf1) gene, which is mutated in the human disease of the same name, is required for rest:activity rhythms in Drosophila. Loss of neurofibromin, the protein product of Nf1, renders flies largely arrhythmic, indicating that it serves an important function in the circadian system. Subsequent studies also reported sleep abnormalities in humans with Nf1 (Johnson et al., 2005; Leschziner et al., 2013; Licis et al., 2013; Maraña Pérez et al., 2015), but these have not been traced to the circadian system, nor have circadian phenotypes been reported in Nf1 mutant mice despite evidence for altered duration of locomotor activity (Weiss et al., 2017). Mouse Nf1 mutants are only available as heterozygotes, because complete loss of Nf1 is lethal in mice; thus, behavioral analysis of the genetic null has not been possible.

Despite their abrogated behavioral rhythms, Drosophila homozygous Nf1 mutants show normal cycling of key circadian clock proteins, period (PER) and timeless (TIM), in central clock cells, indicating that output from the clock is affected (Williams et al., 2001). Consistent with the known function of mammalian neurofibromin as a Ras-GTPase-activating protein (Ras-GAP), effects of Drosophila Nf1 on rest:activity rhythms are mediated by increased Ras/mitogen-activated protein kinase (MAPK) pathway signaling (Williams et al., 2001). However, we still have no understanding of the mechanisms by which Nf1 affects transmission of time-of-day signals from the clock.

Identification of a circadian output circuit provides a unique opportunity to assess the effects of Nf1 on cellular function within cells downstream of the clock. In this circuit, the central clock cells, small ventral lateral neurons (sLNvs), signal via a neuropeptide to effectors of parallel pathways, small lateral neurons (sLNs), and PDF- and sLNv-expressing neurons. Here, we report that Nf1 is required broadly within the Drosophila circadian circuit for rest:activity rhythms and, consistent with that requirement, affects cellular function in multiple neuronal populations. Specifically, loss of Nf1 impairs rhythms of intracellular calcium, a correlate of neural activity, as well as peptide expression in subsets of circadian neurons. Reduction of Nf1 partially rescues rhythms in a PDF receptor mutant, indicating that interactions between neurofibromin and peptide signaling are relevant for behavioral rhythms. As noted earlier, the lethality associated with loss of Nf1 in mice has thus far impeded assessment of its contribution to circadian regulation.

The identification of a cellular endpoint of Nf1 disruption in flies also allowed us to assay the effect of Nf1 loss in mammalian cells, specifically mouse astrocytes that are known to be a site of neurofibromin action (Smithson and Gutmann, 2016). We find that calcium levels are rhythmic over a 24-hr day in wild-type astrocytes, but the cycling is dampened in Nf1-deficient...
Neurofibromin Is Required Broadly in the Drosophila Brain for Rest:Activity Rhythms

We first sought to identify the anatomical location that mediates effects of Nf1 on circadian rhythms. Earlier work, using the upstream activating sequence (UAS)-GAL4 system to target Nf1 expression, indicated that neurofibromin expression in clock neurons was not sufficient for rest:activity rhythms (Williams et al., 2001). Other sites, or perhaps even multiple locations, of action were indicated, which is supported by the finding that Nf1 is expressed broadly across the adult fly brain (Buchanan and Davis, 2010). Thus, we went beyond the clock cell drivers used in the previous study and assayed for rescue of the mutant phenotype following Nf1 expression in different neuronal populations of Nf1 homozygous mutant flies. We found that behavioral rhythms were restored when Nf1 expression was driven by GAL4 drivers expressed broadly, such as the pan-neuronal synaptobrevin-GAL4 driver or the Cha-GAL4 driver expressed in all cholinergic cells (Table 1). Because most adult fly brain neurons are cholinergic (Gorczyca and Hall, 1987), rescue with the Cha driver does not implicate any specific brain region. In addition, the c909-GAL4 and 53b-GAL4 drivers that are expressed in multiple brain regions yielded partial rescue (Table 1).

Consistent with previous findings (Williams et al., 2001), neither the Pdf-GAL4 driver, expressed specifically in ventral lateral neurons (LNvs), nor the Clk4.1-GAL4 driver, which targets DN1s (Guo et al., 2016), rescued the Nf1 mutant phenotype. Rescue was also not seen with expression targeted to monoaminergic cells using the ddc-GAL4 and Tdc2-GAL4 drivers. Even the c929-GAL4 driver, which is expressed in most peptidergic cells, including those of the PI (Hewes et al., 2000), did not rescue the Nf1 mutant phenotype. These results suggest that neurofibromin is required in more than one location for rest:activity rhythms. However, we acknowledge that negative results with some drivers may be due to the reduced strength of these drivers.

Because the rescuing drivers (e.g., synaptobrevin and Cha-GAL4) are expressed at developmental stages, in addition to the adult stage, we asked whether Nf1 is required in adults for normal rest:activity rhythms. We drove expression of Nf1 RNAi with a pan-neuronally expressed and RU-486 inducible driver, synaptobrevin GeneSwitch (nsyb-GS), and knocked down expression specifically in adults by feeding flies RU-486. Knockdown in adults also reduced rhythm strength, although not to the extent seen in Nf1 mutants (Figure S1). The weaker phenotype could reflect incomplete knockdown by RNAi or an additional role in development. Nevertheless, these data indicate that Nf1 functions in adults to maintain circadian behavior.

Neurofibromin Function in Circadian Clock Neurons

Given the rather broad requirement for neurofibromin in the generation of circadian behavioral rhythms, we sought to address its impact in different cells of the circadian circuit important for rest:activity rhythms. We started with the LNvs, which express the PDF neuropeptide. Circadian clock proteins were shown to cycle in LNvs of Nf1 mutant flies (Williams et al., 2001), so we examined effects of neurofibromin loss on rhythmic outputs of these neurons. PDF is the major output of these neurons, and its expression is rhythmic at the terminus of the dorsal projection from the sLNvs (Park et al., 2000). To determine whether this rhythm in PDF expression is intact in Nf1 mutants, we examined expression at times of low and high expression, circadian time (CT) 1 and 13, respectively, in flies maintained in constant darkness (DD) following entrainment to light:dark cycles. Whereas wild-type flies showed a cyclic pattern (Park et al., 2000), PDF expression did not change between time points at the dorsal terminus in Nf1 mutants and was constantly low (Figure 1).

Because reduced accumulation of PDF at the axon terminal is thought to indicate increased peptide release (Park et al., 2000), PDF signaling might be increased in Nf1 mutants. We hypothesized that if Nf1 mutants have increased PDF release, then this could be counteracted by reducing expression of the PDF receptor (PDFR). To test this idea, we generated double mutants of Nf1 loss coupled with each of two mutant alleles of the PDF receptor, pdfr. Like Nf1 mutants, mutants of PDF or of its receptor are largely arrhythmic, although the limited rhythmic flies show a short period (Hyun et al., 2005; Lear et al., 2005; Mertens et al., 2005).

We were unable to rescue rest:activity rhythms of Nf1 homozygous mutants with hypomorphic alleles of pdfr, tested as heterozygotes or homozygotes. This is perhaps not surprising, given that pdfr action in circadian behavior maps to specific neurons, largely the clock network (Im and Taghert, 2010), while neurofibromin, as demonstrated earlier, is required broadly. Nf1 not only is required broadly but also affects multiple circadian cells (described later). However, loss of one copy of Nf1 rescued the short period of both pdfr mutants and partially rescued rhythmicity in one of the two mutant lines (Table 2). Thus, neurofibromin and PDF interact genetically in the generation of behavioral rhythms.

Small LNvs project to DN1 clock cells, which are part of the circuit required for rest:activity rhythms. To determine whether neurofibromin affects the clock or clock output in DN1s, we first examined the expression of the PER clock protein at different times of day in constant darkness. In Nf1 mutants, as in wild-type flies, PER expression was cyclic, peaking at CT1 and nadiring at CT13 (Figure S2). In the absence of an established molecular assay for rhythmic output in these cells, we measured calcium using a CaLexA reporter (Masuyama et al., 2012). Calcium levels were rhythmic in a subset of DN1s, even in constant darkness following entrainment to light:dark cycles, and the rhythm was evident in both wild-type flies and Nf1 mutants (Figure S2). In addition, we did not detect changes in overall calcium levels in Nf1 mutants relative to wild-type controls. We conclude that neurofibromin has limited, if any, effects on DN1s.

Neurofibromin Affects Neural Activity in PI Cells that Secrete DH44

Although cells in the PI do not contain clocks, they receive input from upstream DN1s and are required for different circadian outputs (Barber et al., 2016; Cavanaugh et al., 2014). DH44-positive
neurons in the PI are particularly important for the regulation of rest/activity rhythms (Cavanaugh et al., 2014). To assess a possible role for neurofibron in these cells, we initially assayed DH44 expression in Nf1 mutants. Because DH44 expression does not cycle, we examined a single time point in the presence of light-dark cycles (zeitgeber time 13 [ZT13]) and in constant darkness (CT13) and found that overall expression of DH44 was not different between wild-type flies and Nf1 mutants (Figure 2A) (data not shown). However, this did not preclude an effect of neurofibron on rhythmic output in these cells, which we then assayed by measuring calcium as a proxy for neural activity. As previously reported with a different calcium sensor (Cavey et al., 2016), the CaLexA reporter revealed a rhythm of calcium in these cells, showing significantly higher levels at ZT13 than at ZT1 (Figure 2B). No such rhythm was seen for calcium in DN1 neurons, which are upstream of DH44 cells in the circadian circuit, we surmised that neurofibron is required for rhythmic output from DH44 cells. Because cellular rhythms were normal in DN1 neurons, which are upstream of DH44 cells in the circadian circuit, we surmised that neurofibron had independent effects in DH44 cells.

Table 1. Rescue of Rhythms in Nf1 Mutants by Restoring NF1 in Subsets of Neurons

| Genotype                        | R (%) (n) | Period ± SEM (hr) | FFT ± SEM |
|---------------------------------|-----------|-------------------|-----------|
| n-syb-Gal4,+;UAS-Nf1P1/Nf1P2    | 100 (18/18)| 23.78 ± 0.16      | 0.050 ± 0.006 |
| n-syb-Gal4,+;Nf1P1/P2           | 0 (0/22)  |                   |           |
| Cha-Gal4,+;UAS-Nf1P1/Nf1P2      | 100 (20/20)| 23.77 ± 0.10      | 0.067 ± 0.008 |
| Cha-Gal4,+;Nf1P1/P2             | 4.3 (1/23) | 23.75              | 0.03      |
| Ddc-Gal4,+;UAS-Nf1P1/Nf1P2      | 7.7 (1/13) | 23               | 0.02      |
| Ddc-Gal4,+;Nf1P1/P2             | 0 (0/13)  |                   |           |
| C453/+;UAS-Nf1P1/Nf1P2          | 19.0 (4/21)| 24.12 ± 0.27      | 0.025 ± 0.006 |
| C453/+;Nf1P1/P2                 | 0 (0/13)  |                   |           |
| 1471/+;UAS-Nf1P1/Nf1P2          | 11.1 (2/18)| 24.58 ± 2.08      | 0.03 ± 0.01 |
| 1471/+;Nf1P1/P2                 | 0 (0/13)  |                   |           |
| 201y/+;UAS-Nf1P1/Nf1P2          | 0 (0/32)  |                   |           |
| 201y/+;Nf1P1/P2                 | 0 (0/7)   |                   |           |
| C309/+;UAS-Nf1P1/Nf1P2          | 50 (13/26)| 24.10 ± 0.43      | 0.055 ± 0.006 |
| C309/+;Nf1P1/P2                 | 0 (0/10)  |                   |           |
| 112749/+;UAS-Nf1P1/Nf1P2        | 15.6 (5/32)| 24.10 ± 0.36      | 0.014 ± 0.002 |
| 112749/+;Nf1P1/P2               | 0 (0/16)  |                   |           |
| 53b/+;UAS-Nf1P1/Nf1P2           | 57.1 (16/28)| 24.06 ± 0.21      | 0.024 ± 0.005 |
| 53b/+;Nf1P1/P2                  | 0 (0/13)  |                   |           |
| C739/+;UAS-Nf1P1/Nf1P2          | 0 (0/16)  |                   |           |
| C739/+;Nf1P1/P2                 | 0 (0/3)   |                   |           |
| C929/+;UAS-Nf1P1/Nf1P2          | 0 (0/17)  |                   |           |
| C929/+;Nf1P1/P2                 | 0 (0/11)  |                   |           |
| MJ63/+;UAS-Nf1P1/Nf1P2          | 23.1 (3/13)| 23.83 ± 0.25      | 0.017 ± 0.007 |
| MJ63/+;Nf1P1/P2                 | 0 (0/14)  |                   |           |
| Kurs58/+;UAS-Nf1P1/Nf1P2        | 25.0 (4/16)| 22.96 ± 0.10      | 0.015 ± 0.04 |
| Kurs58/+;Nf1P1/P2               | 8.7 (2/23) | 23.74 ± 0.23      | 0.030 ± 0.07 |
| Clk-1/+;UAS-Nf1P1/Nf1P2         | 8.0 (2/25)| 23.57 ± 0.20      | 0.032 ± 0.004 |
| Clk-1/+;Nf1P1/P2                | 4.5 (1/22) | 23.92              | 0.029     |
| InSITE911/+;UAS-Nf1P1/Nf1P2      | 0 (0/6)   |                   |           |
| InSITE911/+;Nf1P1/P2             | 0 (0/11)  |                   |           |
| 121y/+;UAS-Nf1P1/Nf1P2          | 8.3 (1/12)| 23.83              | 0.036     |
| 121y/+;Nf1P1/P2                 | 0 (0/4)   |                   |           |
| Pdf-G4/+;UAS-Nf1P1/Nf1P2        | 7.6 (1/13) | 23.33              | 0.011     |
| Pdf-G4/+;Nf1P1/P2               | 0 (0/13)  |                   |           |
| UAS-Nf1P1/Nf1P2                 | 8.9 (2/23) | 23.46 ± 0.13      | 0.002 ± 0.000 |

See Figure S1 for adult-specific effects of Nf1 knockdown. Flies showing a fast Fourier transform (FFT) value >0.01 are counted as rhythmic (R). The average periods and FFTs shown represent the mean ± SEM for all rhythmic flies. FFT values were calculated for the first 7 days in constant darkness.
determine whether the effect was cell autonomous, we attempted to rescue the phenotype in DH44 cells by expressing Nf1 specifically in these cells in a mutant background. Calcium cycling was restored in flies expressing Nf1 in DH44 cells, while controls that contained the Nf1 mutation, but only with the Gal4 transgene and not UAS-Nf1, remained arrhythmic. These data indicate that neurofibromin acts in DH44 cells to affect rhythms of calcium.

**Neurofibromin Affects Neural Activity in PI Cells that Secrete SIFamide**

SIFamide (SIFa) is expressed in four PI cells, which are also required for rest:activity rhythms (Cavanaugh et al., 2014). We found that Nf1 loss dramatically increased SIFa mRNA levels (Figure 3A), albeit with a small decrease in the corresponding protein levels as detected in projections throughout the brain (Figures 3B and 3C). Neither RNA nor protein expression was found to cycle, and we did not detect a rhythm in neural activity via calcium imaging. However, Nf1 mutants showed robust increases in calcium levels in SIFa-producing cells, which is likely indicative of increased release and may account for reduced accumulation of protein (Figures 3D and 3E) (Kwon et al., 2015; Park et al., 2000). The strongest influence of neurofibromin loss was observed in SIFa-producing cells. As in the case of DH44 cells, the effects of Nf1 in SIFa cells are likely cell autonomous and independent of those in other circadian cells.

Given the dramatic effects of the Nf1 mutation on SIFa gene expression and SIFa cell activity, we sought to determine whether SIFa contributes to neurofibromin regulation of behavioral rhythms. Although SIFa cells are implicated in circadian rhythms (Cavanaugh et al., 2014), a role for the peptide has not been tested. To examine this potential role, we generated a SIfa mutant by CRISPR/Cas9 genome editing and tested it for circadian rest:activity rhythms. Three independent mutant lines were obtained, and although we observed a small reduction in rhythm strength, this was not consistent across lines and in mutant-deficiency combinations. These results indicate that SIFa is not a major contributor to rest:activity rhythms (Table 3), even though the cells that produce it are important.

SIFa is also implicated in the regulation of sleep (Park et al., 2014), as is Nf1 (Bai and Sehgal, 2015). It is unclear whether effects of Nf1 on sleep are related to its effects on circadian rhythms, but overlapping mechanisms are a distinct possibility given that several circadian neurons have roles in sleep (Artiushin and Sehgal, 2017). We found, as predicted from RNAi analysis (Park et al., 2014), that SIfa mutants have reduced sleep (Figure S3), so we asked whether the Nf1 mutant sleep phenotype is mediated through effects on SIFa expression. We generated double mutants lacking both SIFa and neurofibromin and tested them in sleep assays. Double mutants showed sleep reduction equivalent to that in Nf1 single mutants (Figure S3B), indicating that these two genes act in the same pathway to regulate sleep (Figure S3).

**Neurofibromin Is Required for Calcium Cycling in Mouse Astrocytes**

Neurofibromin has not yet been implicated in circadian rhythms in mammals, although it is associated with sleep phenotypes in humans (Johnson et al., 2005; Leschziner et al., 2013; Licis et al., 2013; Marañá Pérez et al., 2015) and with locomotor activity levels in mice (Weiss et al., 2017). Detection of rhythm phenotypes in knockout mice is complicated by the complete Nf1 loss being embryonic lethal (these mice also frequently show exencephaly) (Lakkis et al., 1999), and effects of the mutation may be
recessive at the cellular level. Based on the preceding studies that indicated a cellular phenotype in Nf1 mutant flies, we sought to determine whether a similar abnormal phenotype was manifest in mammalian cells lacking neurofibromin expression. Because we were interested in adult cells and wanted to avoid the lethality associated with neuronal loss, we used mouse brain-stem astrocytes in which neurofibromin expression was acutely deleted by Cre-mediated excision (Smithson and Gutmann, 2016). Astrocytes were shown to have an important role in circadian timekeeping in the mammalian central clock, the suprachiasmatic nucleus (SCN), so they are a relevant cell type for this purpose (Barca-Mayo et al., 2017; Brancaccio et al., 2017; Tso et al., 2017).

Circadian rhythms can be studied in cultured cells by synchronizing clocks across cells with a stimulus like dexamethasone, which is a synthetic glucocorticoid (Balsalobre et al., 2000); dexamethasone was even used to assay circadian cycling in astrocytes (Barca-Mayo et al., 2017). Typically, Table 2. Nf1P1 Rescues the Short Period Phenotype of the pdfr Mutant

| Genotype               | N  | SR (%) | MR (%) | WR (%) | AR (%) | FFT ± SEM | Period ± SEM (hr) |
|------------------------|----|--------|--------|--------|--------|-----------|-------------------|
| iso31                  | 64 | 87.5   | 9.4    | 1.6    | 1.6    | 0.089 ± 0.004 | 23.79 ± 0.10      |
| Nf1P1/+                | 62 | 48.4   | 33.9   | 9.7    | 8.1    | 0.065 ± 0.004* | 23.72 ± 0.08      |
| pdfr33069/Y            | 32 | 3.1    | 9.4    | 43.8   | 43.8   | 0.026 ± 0.012**** | 22.63 ± 0.15****  |
| pdfr33069/Y;Nf1P1/+    | 32 | 0.0    | 15.6   | 43.8   | 40.6   | 0.022 ± 0.010**** | 23.57 ± 0.06 NS   |
| pdfr33069/Y;Nf1P1/+    | 30 | 16.7   | 16.7   | 46.7   | 20.0   | 0.047 ± 0.009**** | 23.58 ± 0.06 NS   |

SR, strongly rhythmic; MR, moderately rhythmic; WR, weakly arrhythmic; AR, arrhythmic. ****p < 0.0001, *p < 0.05; NS, not significant, compared with iso31. In addition to period, loss of one copy of Nf1 partially rescues overall rhythmicity and rhythm strength of the Pdfr33069 allele.

Figure 2. Neurofibromin Has Cell-Autonomous Effects on Rhythms of Neural Activity in DH44+ PI Neurons

(A) DH44 expression was not significantly different between iso31 and Nf1P1/P2 mutants. The bar graph shows mean ± SEM. n = 5 for iso31, and n = 6 for Nf1P1/P2.

(B) Normalized fluorescence from calcium-dependent GFP expression in DH44-GAL4 > CaLexA/RedStinger flies. The complete genotypes are as follows: control: UAS-RedStinger, lexAop2-mCD8::GFP/DH44-G4; UAS-mLEXA-VP16-NFAT, LexAop-CD2-GFP/+; Nf1P1/P2: UAS-RedStinger, lexAop2-mCD8::GFP/DH44-G4; UAS-mLEXA-VP16-NFAT, LexAop-CD2-GFP, Nf1P1/Nf1P2. Although CaLexA GFP signals in DH44 neurons were not significantly different between control and Nf1P1/P2 flies at either the ZT1 or the ZT13 time point, there was a significant difference in fluorescence intensity between ZT1 and ZT13 in control flies (****p < 0.0001) that was not observed in Nf1P1/P2 flies (one-way ANOVA and Tukey’s multiple comparison test). n = 24–42 cells.

(C) Expression of transgenic Nf1 in DH44 cells of Nf1 mutant flies rescues cycling of calcium. Fluorescence from the CaLexA reporter in DH44 cells was normalized to the mean background intensity of the brain in Nf1 mutants expressing Nf1 in DH44 cells (UAS-RedStinger, lexAop2-mCD8::GFP/+; UAS-mLEXA-VP16-NFAT, LexAop-CD2-GFP, Nf1P1/DH44-Gal4, UAS-Nf1, Nf1P1) and in controls that lacked UAS-Nf1 (UAS-RedStinger, lexAop2-mCD8::GFP/+; UAS-mLEXA-VP16-NFAT, LexAop-CD2-GFP, Nf1P1/DH44-Gal4, Nf1P1). JTK_CYCLE indicated a rhythm in rescued flies. n = 15–26 cells from 4–7 brains per time point.
rhythms of gene expression are assayed in such populations, but we were interested in this case in the levels of calcium over a 24-hr day. Astrocytes were treated with dexamethasone to synchronize circadian cycling, and calcium levels were assayed at different times of day with Cal-520, a recently developed fluorogenic calcium-sensitive dye (Lock et al., 2015). Whereas wild-type astrocytes displayed robust 24-hr oscillations of calcium (Figures 4A and 4B; Figure S3B), this cycling was dampened and the overall levels of calcium were indicative of increased release (Kwon et al., 2015; Park et al., 2018). Our findings suggest that neurofibromin acts broadly in the Drosophila circadian system. Multiple circadian neurons show cellular phenotypes in the Nf1 mutant background, and only drivers with widespread expression rescue the mutant behavioral phenotype. In previous work, we found that clock function was normal in PDF cells, and neurofibromin expression in these cells was insufficient to drive rhythms (Williams et al., 2001; supported also by our current data). As a result, we did not examine the cycling of PDF at the dorsal termini of sLNvs. We now report that this cycling is altered, suggesting that Nf1 affects outputs of the clock within PDF cells. The behavioral relevance of rhythmic PDF accumulation has been questioned (Kula et al., 2006), but subsequent studies have supported a role for cellular rhythms at sLNv termini (Fernández et al., 2008; Helfrich-Förster et al., 2000). Loss of Nf1 abrogates these cellular rhythms, in conjunction with loss of PDF cycling. PDF expression at sLNv termini is also reduced, which is typically indicative of increased release (Kwon et al., 2015; Park et al., 2000) and consistent with increased MAPK activity in this region of the brain in Nf1 mutants (Williams et al., 2001). Effects of Nf1 mutants on rhythms are mediated through increased MAPK activity (Williams et al., 2001), which is also a

**DISCUSSION**

Although the effects of neurofibromin on Drosophila circadian rhythms were reported more than 15 years ago, the cellular basis of action has remained elusive (Williams et al., 2001). Analysis of other circadian output molecules has also been difficult due to lack of a conceptual framework for output circuitry. Therefore, the identification of neurons downstream of the central clock (Cavanaugh et al., 2014) allows us to examine the function of output molecules in a cellular context. Leveraging these insights, we show that neurofibromin loss affects neural activity, as well as gene expression, in peptidergic neurons of a circadian circuit required for rest:activity rhythms. The demonstration that neurofibromin is similarly required for calcium-cellular activity rhythms in mouse astrocytes provides evidence for a conserved function of neurofibromin in the generation of rhythms.
major target of neurofibromin in mammals (Rad and Tee, 2016). Conversely, Pdf mutants show decreased MAPK activity. In addition to affecting rhythmic outputs of the sLNvs, neurofibromin is implicated in signaling through the PDFR (Mertens et al., 2005). The impact of neurofibromin on PDF signaling is relevant to behavioral rhythms, because rhythm phenotypes of pdf are partially rescued by a reduction in Nf1 gene expression. We infer that increased PDF signaling caused by loss of one copy of Nf1 compensates for reduced pdfr expression (pdfr mutants do not appear to be nulls) (Hyun et al., 2005; Lear et al., 2005; Mertens et al., 2005). Nf1 mutants are not rescued by a reduction in pdfr, most likely because Nf1 action at multiple sites contributes to the behavioral phenotype. Alternatively, lack of rescue may primarily reflect a need for neurofibromin action downstream of pdfr.

Clock function and neural activity are normal in DN1s of Nf1 mutant flies. Although we cannot exclude other effects in DN1s—for instance, the increased MAPK activity mentioned on downstream PI cells. More likely, the PI phenotypes in Nf1 mutant flies represent additional sites of neurofibromin action and are not secondary to upstream defects in the pathway. This interpretation is supported by cell-autonomous rescue of the phenotype in DH44 cells. It is also consistent with the broad requirement for neurofibromin in the rescue of behavioral rhythms and the lack of rescue of the Nf1 behavioral phenotype by pdfr mutants.

Dampened cycling of neural activity in DH44 cells of the PI is likely an important contributor to the arrhythmic phenotype, because these cells are critical for circadian behavioral rhythms (Cavanaugh et al., 2014; King et al., 2017). The phase of calcium activity is the same as that reported previously (Cavey et al., 2016) and consistent with a role for DH44 in the evening peak of locomotor activity (King et al., 2017). An even stronger phenotype is observed in Nf1 mutants in cells that produce SIFa. These cells show very high activity and higher levels of SIFa mRNA, although the levels of protein are not elevated. We suggest that the protein does not accumulate because it is constantly secreted in response to the high neural activity; mRNA levels may increase to compensate for the continually depleted protein. Nf1 phenotypes in circadian cells indicate not only a cellular mechanism of action but also potential molecular targets (PDF and SIFa). Genetic interactions of Nf1 with pdfr in the context of circadian rhythms and with SIFa in the regulation of sleep suggest that these molecular targets are relevant for behavior. We suggest that effects of neurofibromin loss on sleep result from dysregulated SIFa signaling. Effects of Drosophila Nf1 on peptidergic signaling are supported by a former report showing rescue of synaptic growth phenotypes in Nf1 flies by a mutation in a peptidergic receptor (Walker et al., 2013).

To extend our findings of aberrant calcium signals and neural activity in Nf1 mutant flies to higher organisms, we sought to determine whether these phenotypes were conserved in mammals. Herein, we show that astrocytes display rhythmic calcium cycling, likely reflecting rhythmic cellular activity, and that these rhythms are attenuated in Nf1+/-- cells. Increased excitability and secretion phenotypes were found in Nf1-deficient mouse cells (Hingtgen et al., 2006; Wang et al., 2005), and although the mechanisms are still unclear, they may involve post-translational modification of N-type calcium channels (Duan et al., 2014). Given that neurofibromin is a potent regulator of MAPK activity, phosphorylation is a candidate mechanism for such modifications. However, effects of Nf1 on cellular activity or excitability have not been studied in the context of rhythms. In addition, as indicated earlier, assays of behavioral rhythms in Nf1 mutant mice have been complicated by the lethality of the null mutant.

The extent of neurofibromin loss in Nf1 patients tends to vary considerably. Homozygous Nf1 loss in human brain neurons has not yet been reported, but studies have demonstrated that not all germline Nf1 gene mutations are equivalent, with some leading to dramatic reductions in neurofibromin expression (>80%) and others exhibiting more modest effects (Anastasaki et al., 2015). Recapitulating these mutational differences in mice produces variations in the penetrance and severity of phenotypes (Toonen et al., 2016), which is likely also the case in humans. It is possible that circadian rhythm abnormalities would only be observed in individuals with strong hypomorphic germline mutations in the Nf1 gene. Nevertheless, deficits in sleep initiation and maintenance have been reported in children with neurofibromatosis (Johnson et al., 2005; Leschziner et al., 2013; Licis et al., 2013;}

| Genotype | N  | SR (%) | MR (%) | WR (%) | AR (%) | FFT ± SEM | Period ± SEM (hr) |
|----------|----|--------|--------|--------|--------|-----------|------------------|
| iso31    | 16 | 87.5   | 6.3    | 0.0    | 0.0    | 0.133 ± 0.016 | 23.83 ± 0.03     |
| SIFa+/+  | 11 | 100.0  | 0.0    | 0.0    | 0.0    | 0.140 ± 0.014 | 23.85 ± 0.05     |
| SIFa1    | 15 | 26.7   | 33.3   | 26.7   | 0.0    | 0.055 ± 0.011 | 24.04 ± 0.09     |
| SIFa+/Def| 16 | 56.3   | 18.8   | 25.0   | 6.7    | 0.059 ± 0.011 | 24.59 ± 0.17     |
| SIFa2+/+ | 8  | 100.0  | 0.0    | 0.0    | 0.0    | 0.149 ± 0.014 | 23.84 ± 0.08     |
| SIFa2/Def| 16 | 86.7   | 13.3   | 31.3   | 0.0    | 0.100 ± 0.013 | 23.89 ± 0.07     |
| SIFa3+/+ | 5  | 100.0  | 0.0    | 0.0    | 0.0    | 0.120 ± 0.006 | 23.83 ± 0.03     |
| SIFa3/Def| 16 | 68.8   | 12.5   | 6.3    | 12.5   | 0.092 ± 0.013 | 23.94 ± 0.04     |
| SIFa3/Def| 16 | 43.8   | 6.3    | 50.0   | 0.0    | 0.059 ± 0.010 | 24.86 ± 0.12     |

SR, strongly rhythmic; MR, moderately rhythmic; WR, weakly arrhythmic; AR, arrhythmic.
MARANA PÉREZ ET AL., 2015) and could have their basis in the mechanisms we report here.

EXPERIMENTAL PROCEDURES

Fly Lines
The GAL4 lines were used previously in the lab (Bai and Sehgal, 2015; Cavanaugh et al., 2014; Crocker et al., 2010). Nf1P1, Nf1P2, and UAS-Nf1 lines were reported (Williams et al., 2001) and were outcrossed into an iso31 background for 7 generations. Bloomington line Df27352 was used as an SIFα deficiency allele (2R:60C2;60D14) tested in transheterozygotes with SIFα CRISPR alleles. CalExA flies were a gift from J. Wang. UAS-RedStinger flies were obtained from the Bloomington Drosophila Stock Center.

Generation of Nsyb–GS Flies
nSyb-GS construct was generated using Gateway cloning (Invitrogen) and the construct was targeted to the PhiC31-attP2 landing site on the third chromosome (3L:68A4) in Drosophila. A 1,917 bp fragment of the Drosophila n-synaptobrevin (Nsyb) promoter (matching the fragment used to generate Nsyb–GAL4 flies by Julie Simpson, Janelia Farm Research Campus) was PCR amplified from Canton-S flies and recombined into the Gateway pDONR221 vector using primers containing Gateway attB1/2 sites: attB1_Nsyb1917_5′, 5′-GGGGACA

Figure 4. Genetic Ablation of Nf1 Upregulates Calcium Levels and Disrupts Calcium Oscillations in Mammalian Astrocytes
(A) Fluorescence images of a time course of changes in calcium in wild-type (control) and Nf1+/− (MUT) astrocytes after dexamethasone stimulation. Images were taken at low magnification (40×) using a FITC filter set (474 nm excitation and 530 nm emission). Scale bar, 50 μm. See Figures S4A and S4B for detailed experimental procedures and higher-magnification images.
(B and C) Quantitative measurement of calcium changes in ten wild-type (control) astrocytes (B) or Nf1+/− (MUT) astrocytes (C) at the time points indicated. The results are plotted as line graphs. See Figure S4C for details of quantitative calcium measurements in single cells.
(D) Quantitative comparison of changes of calcium levels over time in wild-type (control) and mutant (MUT) astrocytes. Error bars represent SD of the mean. **p < 0.05, ***p < 0.0005.
Figure S4D provides statistical analysis of time-of-day effects, using JTK_CYCLE and Lomb-Scargle algorithms.
The intensity was measured. Nuclear RedStinger expression is calcium independent CaLexA signals, each cytoplasmic area was manually selected and mean in-average background staining level for the boxed region. The integrated intensity termini, the terminus region was manually selected as a box, including the All images were analyzed with ImageJ software. For PDF intensity at sLNv dor-

Image Analysis
All images were analyzed with ImageJ software. For PDF intensity at slNv dor-
sal termini, the terminus region was manually selected as a box, including the axon terminus upward from the branching point, and then thresholded with the average background staining level for the boxed region. The integrated intensity was measured and compared between genotypes. For quantification of CalExA signals, each cytoplasmic area was manually selected and mean intensity was measured. Nuclear RedStinger expression is calcium independent and was used to normalize the GFP signals, except in Figure 2C, where the GFP signals were normalized to the mean background intensity of the brain.

Sleep Assays
Sleep was monitored with the single-beam Drosophila Activity Monitoring System (Trikinetics, Waltham, MA) or, when indicated, with multi-beam monitors (Trikinetics, Waltham, MA) as described previously (Bai and Sehgal, 2015).

Rest/Activity Rhythm Analysis
Locomotor activity assays were performed with the Drosophila Activity Monitoring System (Trikinetics, Waltham, MA) as described previously (Cavanaugh et al., 2014). Individual male flies were loaded into glass tubes containing 5% su-

Preparation of Entry Vectors for Geneswitch
To verify induction of UAS expression with RU-486, Nsyb-GS flies were crossed to UAS-mCD8:

Generation of SIFa Mutants
The SIFa mutants were generated with CRISPR/Cas9-mediated genome editing (Port et al., 2014). Tandem SIFa-targeting guide RNA sequences flanking the SIFa coding region were introduced into pCASF4 (Addgene plasmid 49411) using the primers SIFa-KO-Forward (5′-TATATAGGAGATATCCGGGTGAACTTCGTAggctcgagatcagttctGTTTTAGAGCTAGAAAT

Immunohistochemistry
Adult fly brains were dissected in cold PBS with 0.1% Triton-X (PBST) and fixed in 4% formaldehyde for 15–20 min on ice. Brains were rinsed 3 × 10 min with PBST, blocked for 30–60 min in 5% normal donkey serum in PBST (NDST), and incubated overnight (ON) at 4 °C in primary antibody diluted in NDST. Brains were then rinsed 4 × 10 min in PBST, incubated 2 hr in secondary antibody diluted in NDST, rinsed 4 × 10 min in PBST, and mounted with Vectashield. The following primary antibodies were used: rabbit anti-GFP 1:1,000 (Molecular Probes A-11122), rabbit anti-DH44 1:500 (John-

Rhythms in Astrocyte Cultures
Wild-type and Nf1−/− astrocyte cultures were generated as previously described (Smithson and Gutmann, 2016). Brainstem astrocytes obtained from Nrfl2−/− mouse pups (postnatal days 1–2) were seeded with adenovirus 

Electrophysiology
Rhythms in Astrocyte Cultures
Wild-type and Nf1−/− astrocyte cultures were generated as previously described (Smithson and Gutmann, 2016). Brainstem astrocytes obtained from Nrfl2−/− mouse pups (postnatal days 1–2) were seeded with adenovirus 

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AUTHOR CONTRIBUTIONS

Conceptualization, L.B., X.Z., and A.S.; Methodology: L.B., Y.L., D.C., and D.H.G.; Investigation, L.B., Y.L., C.T.H., J.A.W., D.C., X.Z., and H.W.; Resources, C.S., P.H., and D.H.G.; Writing – Original Draft, L.B. and A.S.; Writing – Reviewing & Editing, A.S., L.B., D.H.G., J.A.W., and Y.L.; Funding Acquisition, A.S. and D.H.G.; Supervision, A.S.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Supplemental Information

A Conserved Circadian Function
for the Neurofibromatosis 1 Gene

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Supplemental Figure 1 (Related to main Table 1). RNAi-mediated knockdown of Nf1 with an inducible pan-neuronal driver (Nsyb-GS) alters circadian rhythms. A. Representative actograms of individual flies of indicated genotypes. Locomotor activity was recorded in constant darkness for 9-11 days as shown. Males fed vehicle (-) or RU-486 (+) are shown in the top panels and females are in the lower panels. B. Period length as measured by Chi² and relative FFT power are reported for males and females of indicated RNAi lines with (+) and without (-) RU-486. Comparisons were between (+) and (-) groups; * =p< 0.05, ** = p<0.009, and *** =p<0.0001, student’s t-test.
Supplemental Figure 2 (Related to main Figure 1). DN1 clock cells display normal molecular cycling and activity patterns in Nf1 mutant flies. (A) PER protein staining in the DN1 neurons in constant darkness. Representative images of 7-8 brains per time point are shown in the top panel. Average DN1 fluorescence intensity per brain was plotted as mean±SEM. Iso31 and Nf1^{P1/P2} were not significantly different at any time point (One-way ANOVA and Tukey’s multiple comparison tests). (B) Calcium-dependent GFP expression in InSITE 911-GAL4>UAS-CaLexA/UAS-RedStinger flies. All DN1 neurons were labeled with nuclear RedStinger. Images are representative of 8-12 brains. The number of DN1 cells with high fluorescence (normalized to the RedStinger signal, ≥0.50) was significantly higher at ZT1 than at ZT13 in both control and Nf1^{P1/P2} flies (One-way ANOVA and Tukey’s tests). Bar graph shows mean and SEM. N=8-12 brains. (C) Quantification of calcium-dependent GFP expression in all DN1 cells detected in wild type and mutant flies at ZT 1. Normalized GFP intensity was determined for individual DN1 neurons. Lines and error bars are mean and SEM, respectively. One-way ANOVA and Tukey’s multiple comparison test did not detect any difference between control and Nf1^{P1/P2} at either time point, when fluorescence in all DN1s was taken into account. Thus, DN1s are similar at ZT1 and ZT13 in terms of overall calcium signal, but the number of DN1s with high calcium-dependent fluorescence (≥ 0.50) is greater at ZT1 (shown in B) in both control and Nf1^{P1/P2} flies.
**Supplemental Figure 3 (Related to main Figure 3).** *SIFa* mutants have reduced sleep. (A) Total sleep is shown for males and females (mean±SEM) in wildtype control, *SIFa* heterozygous and *SIFa* homozygous fly lines. N=5-16 for males and 12-16 for females. Significant differences were found with one-way ANOVA analysis and Tukey’s multiple comparison tests. *indicates significant difference from control iso31 flies. *P*<0.05; ****P<0.0001. (B) Data for double mutants of *SIFamide* and *Nf1* are shown. *Nf1* is epistatic to *SIFa* in the regulation of sleep. Total sleep measured with multi-beam monitors is shown as mean±SEM. Groups with the same alphabetic label on top are not statistically different from each other; groups with different labels are statistically different (P<0.05). While both *SIFa*2/3 and *Nf1*P1/P2 mutants have reduced sleep compared to iso31, *SIFa*2/3; *Nf1*P1/P2 double mutants do not show a further sleep decrease relative to *Nf1*P1/P2 flies. N=8-16. (C) Schematic representation of *Nf1* function in the circadian circuit. The cellular phenotype observed in each cell type is indicated. As *SIFa* and *Nf1* may affect sleep through the same pathway, sleep is noted as a possible phenotype associated with *SIFa* cells.
A

Dex (1µM) treatment at 4 hr intervals

Cal 520 (4µM) treatment at 4 hr intervals

Sampling after 2hr treatment of Cal 520

24 hr 48 hr 72 hr

Control

Nfl-/-

B

Time after dex stimulation

24h 28h 32h 36h 40h 44h 48h 52h 56h 60h 64h 68h 72h

Control

Nfl-/-

Dapi-merge Cal 520

Dapi-merge Cal 520

C

475 nm (Ex) / 530 nm (Em)

D

JTK cycle analysis results

| CycID   | BH.Q        | P     | Period | Phase | Amplitude     |
|---------|-------------|-------|--------|-------|---------------|
| Control | 1.07E-20    | 5.36E-21| 24     | 14    | 24.1399184    |
| Nfl-/-  | 0.01945527  | 0.01945527| 24     | 8     | 8.650744361   |

LS analysis results

| CycID   | BH.Q       | P     | Period | Phase |
|---------|------------|-------|--------|-------|
| Control | 2.60E-12   | 1.30E-12| 24.0   | 36.3  |
| Nfl-/-  | 0.26       | 0.25923774 | 23.4   | 36.1  |
Supplemental Figure 4 (Related to main Figure 4). *Nfl* deficiency alters normal circadian oscillations of calcium. (A) Schematic depiction of the calcium cycling assay of mammalian astrocytes. 24 hr after 1µM dexamethazone (Dex) treatment of control and *Nfl* deficient cells (*Nfl*-/− astrocytes), the cells were incubated with Cal 520 (4µM) solution for 2 hours before sampling at the time points indicated for two days. (B) Highly magnified fluorescence images of a time-course of changes in calcium in wild-type (Control; upper panels) and *Nf1*-/− astrocytes (lower panels) after dex stimulation. Images were taken with FITC (474nm excitation and 530 nm emission) and Dapi (350nm excitation and 470nm emission) filter sets. Dapi-merged images are shown in parallel to indicate cell nuclei. Scale bar: 20µm (C) Quantitative measurement of calcium in individual wild-type (Control) or *Nf1*-/− astrocytes shown in Figure 4. Original green fluorescent images of cells were converted to white and black mode, cells with calcium signals were demarcated (yellow dashed lines) and the intensity of the signal was quantified using Image J software. (D) JTK_CYCLE and Lomb-Scargle (LS) analysis of the calcium cycling dataset shown in Figure 4 using Meta-cycle R program (Wu et al., 2016). JTK cycle analysis revealed a significant rhythm in wild type (P: 5.36E-21) and *Nf1* (P: 0.01945) samples, but an advanced phase and lower amplitude in the latter (24.1399 in wild type versus 8.65 in *Nf1*). LS only detected a rhythm in wild type, but not in *Nf1* mutant flies. BH.Q: q-value estimated by the Benjamini-Hochberg procedure.