Supporting Information

Webb et al. 10.1073/pnas.0902768106

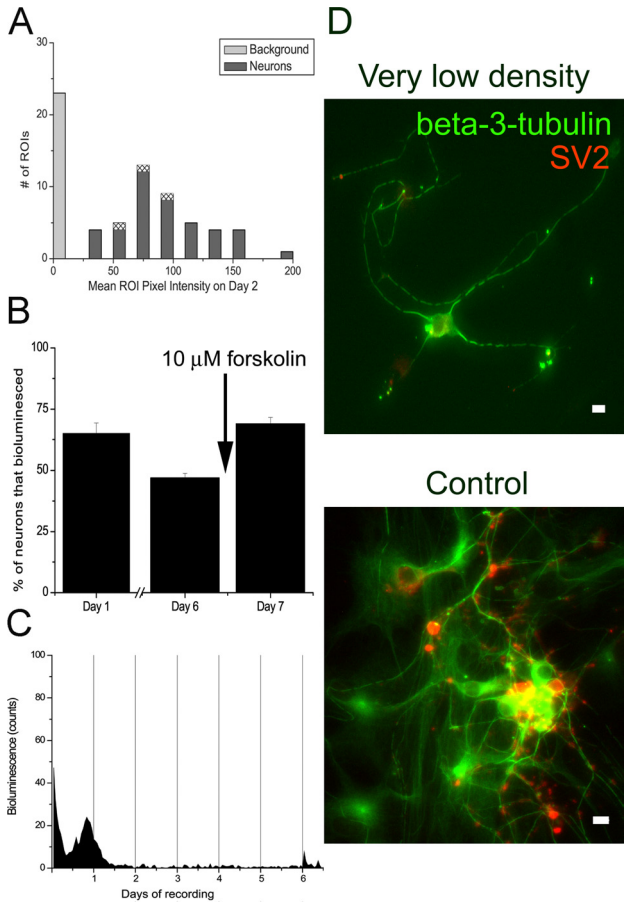

Fig. S1. Neurons in very-low-density dispersals are healthy, but most do not express markers for intercellular communication. In addition to assessments by whole-cell patch recording, multielectrode array recording of action potentials, and bright field and DAPI imaging (Figs. 1, 2, and 4), we tested the viability of low-density cultures after 6 days of recording using their response to stimulation and their neuropeptide expression. (*A*) To determine a threshold bioluminescence level for nonexpression, we compared maximum pixel intensity during the second 24 h of recording from rhythmic (dark gray) and arrhythmic (white crosshatch) bioluminescent neurons (n = 47) or background areas with no cells (n = 23, light gray). In these experiments, we found 134 cells that luminesced well above background levels but were clearly not circadian. Subsequently, we defined cells as nonexpressing when their maximum bioluminescence was below 5 counts per 24 h. (*B*) Forskolin restored the percentage of bioluminescent neurons to the level seen on the first day of the recording (65% vs. 69% of 241 neurons recorded in 2 cultures). (*C*) Induced and nonexpressing neurons also stain for neuropeptides. A representative VIP-ergic neuron within the field shown in Fig. 1 *A* briefly expressed PER2, as can been seen in its bioluminescence trace. We treated all cultures with colchicine before fixation to diminish concern for circadian variations in the detectability of neuropeptide content. We found that the percentage of VIP- and AVP-positive neurons was similar in high-density and very-low-density cultures and in low-density cultures fixed between 10 and 15 days after plating. We conclude that the proportion of each class of peptidergic neuron was relatively stable over the course of the experiment. (*D*) Immunolabeling for β-3-tubulin, a marker for axons and dendrites, and for the presynaptic protein SV2, revealed no evidence for intercellular communication in very-low-density cultures (*Top*) and many sites of apposition in control cultures (*Bottom*). A few neurons in very-low-density cultures formed apparent synapses on themselves (autapses), consistent with the rare inhibitory-like currents recorded in these cultures. Cultures of primary cortical astrocytes showed no SV2 staining, similar to neurons plated at very low densities. (Scale bars, 10-μm.)

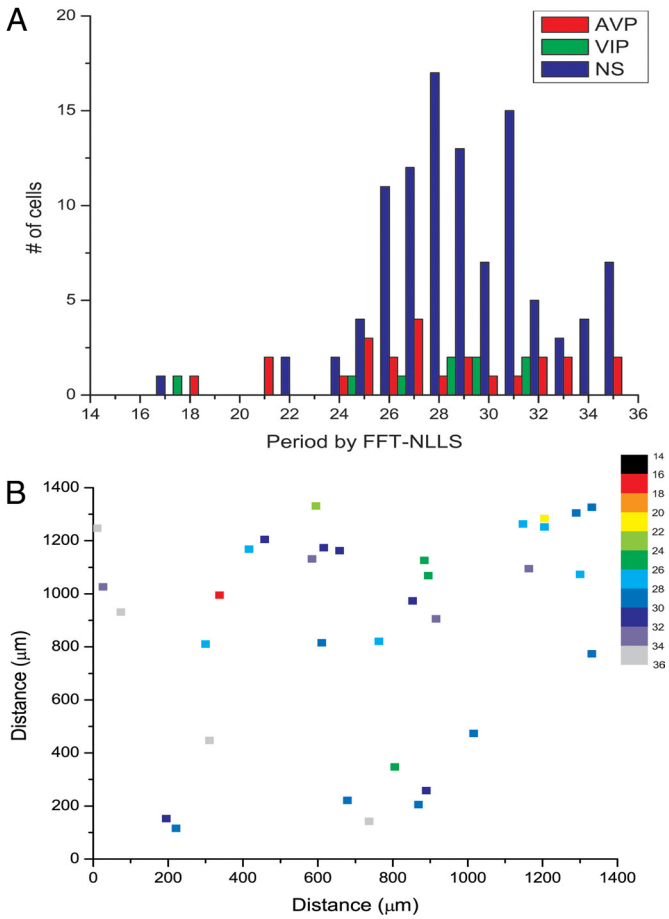

Fig. S2. Very-low-density dispersals show no evidence for circadian coupling. (*A*) Periods as measured by FFT-NLLS from AVP-ergic, VIP-ergic, or nonstained neurons ($n = 139$ of 555 recorded and stained cells in 5 cultures) were broadly distributed and did not differ between neurochemical classes (Forsythe and Brown-Levine's tests, $P > 0.05$). (*B*) Neurons ($n = 34$) in a representative very-low-density culture show uncorrelated periods ranging from 14 to 36 h. The period of each circadian neuron is plotted at the location of its soma in hours according to the pseudocolored scale bar. Neighboring cells tended to have dissimilar periods (regression analysis comparing location with period value, $P > 0.05$), suggesting a lack of circadian signaling between cells within these cultures.

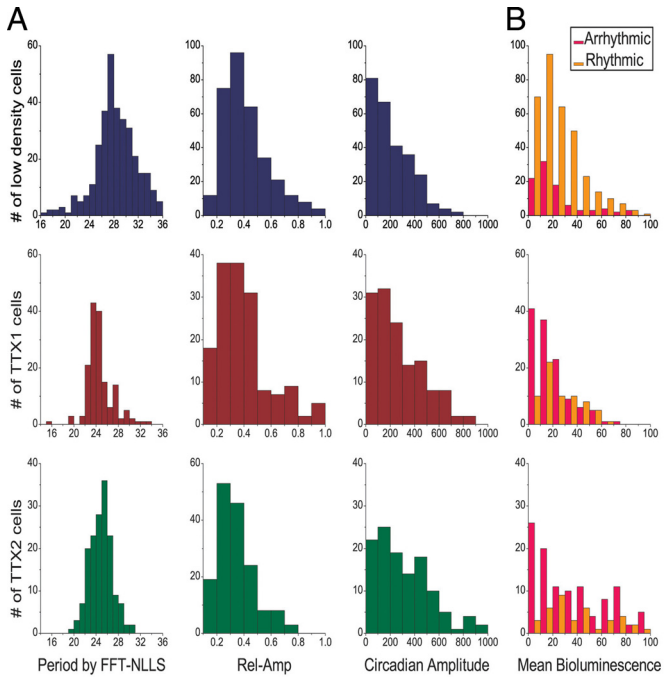

Fig. S3. Characteristics of PER2::LUC expression in single cells provide evidence for a continuum of oscillator phenotypes among SCN neurons, rather than distinct pacemaker classes. We compared circadian properties and mean PER2 expression of SCN neurons in very-low-density dispersals ($n = 252$ neurons of 1,413 recorded in 14 cultures) and in SCN explants during the first (TTX1) and second (TTX2) treatments with tetrodotoxin (TTX1, $n = 158$ of 190 recorded in 2 explants; TTX2, $n = 161$ of 190 recorded in 2 explants). (*A*) Circadian neurons recorded in each of the 3 conditions had broadly distributed circadian properties, including period, rel-amp (the ratio of the amplitude error to the most probable amplitude magnitude from a fast-Fourier transform-nonlinear least-squares fit) (1), and circadian amplitude (the power above the 95% confidence interval at the dominant period in a χ^2 periodogram) (2). Thus, we found no evidence for clustering of properties among circadian neurons. (*B*) The distributions of mean bioluminescence also failed to reveal any differences between rhythmic and arrhythmic neurons in these 3 culture conditions.

1. Plautz JD, et al. (1997) Quantitative analysis of *Drosophila* period gene transcription in living animals. *J Biol Rhythms* 12:204–217.
2. Sokolove PG, Bushell WN (1978) The chi square periodogram: Its utility for analysis of circadian rhythms. *J Theor Biol* 72:131–160.

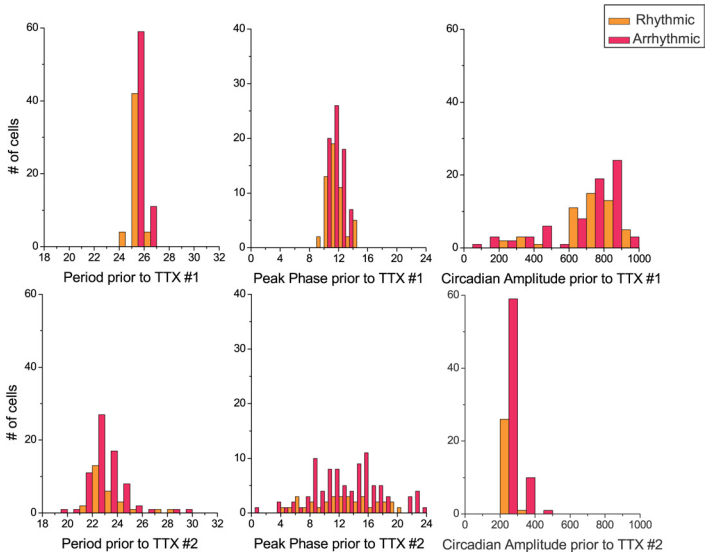

Fig. S4. Period, phase, and circadian amplitude before TTX treatment do not predict whether a neuron will remain circadian in TTX. Period distribution of PER2::LUC expression for 6 days before the first (*Top*) and second (*Bottom*) TTX treatments for 123 neurons that were either rhythmic (pink) or arrhythmic (orange) shows no correlation of circadian behavior with prior period. Peak phase or circadian amplitude of PER2::LUC expression in the 24-h cycle before TTX application also did not correlate with outcome, strongly indicating that each cell's phase or amplitude of oscillation at the time of TTX treatment did not determine whether they would lose rhythmicity.

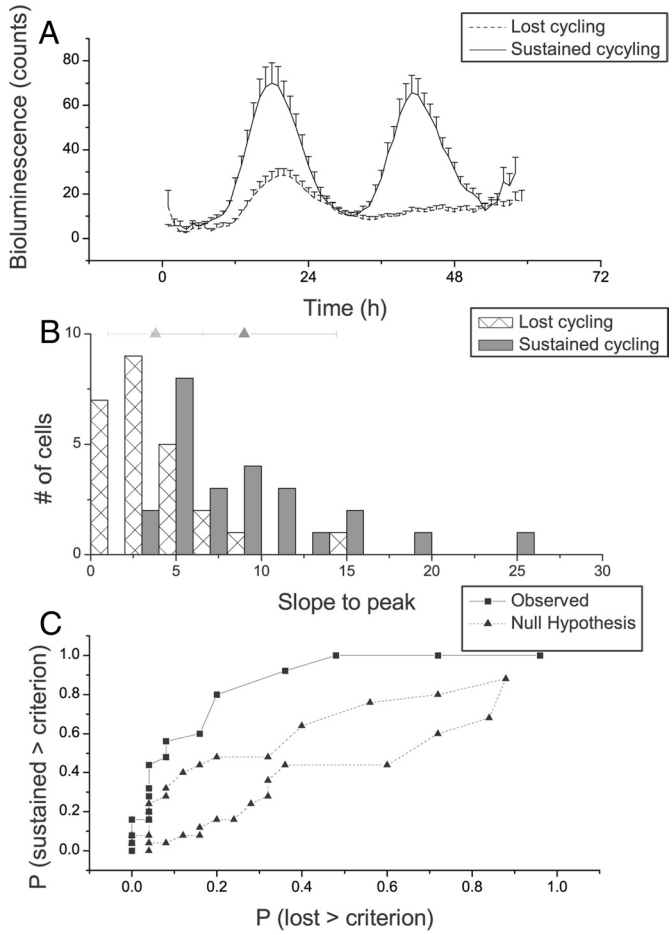

Fig. S5. Rate of PER2 accumulation is a reliable predictor of changes in oscillatory behavior in single SCN neurons. (*A*) The average waveforms of neurons that sustained (solid line) or lost (dashed line) circadian expression of PER2 show that the bioluminescence of neurons that failed to continue cycling was of lower amplitude and slower to rise on the cycle prior. Error bars show the SEM of 25 randomly selected neurons in each group. (*B*) The distributions of slope of the rising phase of PER2::LUC expression differed between neurons that lost rhythmicity (white crosshatch) and neurons that continued to cycle (dark gray). Mean and SD are plotted for both groups. (*C*) ROC curve of 50 neurons whose average waveforms are represented in *A* (circles). To calculate each point along the ROC curve, the proportion of rhythmic neurons that exceeded a given slope criterion were plotted against the proportion of neurons that lost cycling whose slope exceeded the same criterion. For example, for a criterion slope of 4 (i.e., bioluminescence increased by at least 4 counts per hour from the trough to the peak of the first cycle), we found that 23 of 25 neurons sustained rhythmicity (0.92) and 9 of 25 neurons lost cycling (0.36). Random sampling of the same slope data established a 95% confidence interval (triangles) of the null hypothesis that the slope does not predict rhythmicity in the next cycle. According to this ROC analysis, an ideal observer could accurately predict the behavior of an SCN neuron 83% of the time using the slope of PER2 accumulation. The ROC curve significantly differed from those generated from randomly sampled data (permutation test, $P < 0.001$). Interestingly, the peak-to-trough amplitude predicted whether a neuron would remain circadian only 71% of the time. Finally, we examined the slope of PER2::LUC expression from rhythmic neurons in untreated SCN explants and found that their average slope was 16 times greater than that of TTX-treated neurons that lost rhythmicity. We conclude that the rate of PER2 accumulation in one cycle is a reliable predictor of circadian behavior on the subsequent cycle. These results are consistent with in vivo observations of gain or loss of circadian rhythmicity. For example, locomotor and melatonin patterns lose and regain near 24-h cycling in arctic animals exposed to seasonal changes in day length (1). Food restriction for a few hours each day induces daily oscillations in the dorsomedial hypothalamus of mice (2). Clock gene expression is noncircadian in the liver of voles until they are provided with a running wheel or daily feeding cycles (3).

1. van Oort BE, et al. (2005) Circadian organization in reindeer. *Nature* 438:1095–1096.
2. Fuller PM, Lu J, Saper CB (2008) Differential rescue of light- and food-entrainable circadian rhythms. *Science* 320:1074–1077.
3. van der Veen DR, et al. (2006) Impact of behavior on central and peripheral circadian clocks in the common vole *Microtus arvalis*, a mammal with ultradian rhythms. *Proc Natl Acad Sci USA* 103:3393–3398.

Table S1. Comparison of the predicted and observed probabilities of rhythmicity and neuropeptide expression

Of 555 total neurons	Circadian behavior		Neuropeptide expression	Probability	Observed
Rhythmic	109			0.033	0.032
		AVP	93	0.016	0.012
		VIP	46	0.15	0.15
Arrhythmic	446	Other	416	0.13	0.14
				0.07	0.07
				0.60	0.60

To determine the predicted joint probability assuming that circadian cycling is independent of AVP or VIP expression, we calculated the chance that a neuron was rhythmic out of 555 recorded-and-stained neurons, and then multiplied that fraction by the chance that a neuron expressed a given neuropeptide. We found that these probabilities were similar to the observed likelihoods that a neuron was circadian and expressed AVP, VIP, or neither. For example, 3.2% of the recorded neurons were circadian and expressed AVP. This is consistent with the prediction that, of SCN AVP-ergic neurons (93 of 555 or 17% of SCN neurons), 20% (109 of 555) will show intrinsic daily rhythms at any given time ($0.17 \times 0.20 = 0.033$ or 3.3%).