unimodal or arrhythmic behavior patterns under constant darkness (26) (fig. S1I and table S2). In these flies, we found that the Ca\textsuperscript{2+} rhythms in M cells (s-LNv and DN1) were unaffected, but they were phase-shifted in LN\textsubscript{d} and DN\textsubscript{3}, such that these two groups now produced Ca\textsuperscript{2+} rhythms around dawn, roughly in synchrony with M cells (Fig. 4, A and B). The phase of I-LN\textsubscript{v} did not change, consistent with the absence of PDF sensitivity by this pacemaker group (27). The phase shifts in LN\textsubscript{d} and DN\textsubscript{3} were fully restored by the expression of complete pdfr from a bacterial artificial chromosome (BAC) transgene (Fig. 4, C to E, “rescue 1,” and fig. S1I). Thus, PDF, which promotes synchronization of molecular clocks under constant conditions (10, 28), is also needed to properly stagger their Ca\textsuperscript{2+} activity phases across the day. Whether the phases of the I-LN\textsubscript{v} and DN\textsubscript{1} are set by other intercellular signals remains to be determined.

We further examined the pdfr mutant phenotype at higher cellular resolution [pdfy8- GCaMP6s; Fig. 2A). The PDFR-expressing E cell groups (the three PDFR-expressing LN\textsubscript{d} and the fifth s-LN\textsubscript{v}) displayed phase shifts similar to those of the entire LN\textsubscript{d} group (Fig. 4, F and G). When pdfR expression was restored just in these subsets of pacemaker neurons (with GAL4-UAS), both behavior and Ca\textsuperscript{2+} rhythms were partially restored (Fig. 4, H to J, “rescue 2,” and fig. S1I and table S2). The phase of the fifth s-LN\textsubscript{v} was fully restored, which suggests that PDFR signaling is required for cell-autonomous setting of Ca\textsuperscript{2+} phase in this pacemaker group. However, in rescue 2, a single LN\textsubscript{d} typically remained active around dawn, whereas two LN\textsubscript{d}s were active around dusk (fig. S12), which we interpret as a partial restoration or a nonautonomous phase-setting mechanism for LN\textsubscript{d}.

Our results show that molecular clocks drive circadian rhythms in the neural activity of pacemakers. Temporally patterned neural activity encodes different temporal landmarks of the day in a manner that reflects the different functions of the pacemaker groups. The homogeneous molecular clock produces sequential activity peaks by a mechanism dependent on PDFR signaling. By generating diverse phases of neural activity in different pacemaker groups, the circadian clock greatly expands its functional output.

SYNAPTIC VESICLES

Single-vesicle imaging reveals different transport mechanisms between glutamatergic and GABAergic vesicles

Zohreh Farsi,1 Julia Preobraschenski,2 Geert van den Bogaart,2 Dietmar Riedel,3 Reinhard Jahn,1,4,5 Andrew Woehler4,5

Synaptic transmission is mediated by the release of neurotransmitters, which involves exo-endocytic cycling of synaptic vesicles. To maintain synaptic function, synaptic vesicles are refilled with thousands of neurotransmitter molecules within seconds after endocytosis, using the energy provided by an electrochemical proton gradient. However, it is unclear how transmitter molecules carrying different net charges can be efficiently sequestered while maintaining charge neutrality and osmotic balance. We used single-vesicle imaging to monitor pH and electrical gradients and directly showed different uptake mechanisms for glutamate and \(\gamma\)-aminobutyric acid (GABA) operating in parallel. In contrast to glutamate, GABA was exchanged for protons, with no other ions participating in the transport cycle. Thus, only a few components are needed to guarantee reliable vesicle filling with different neurotransmitters.

All synaptic vesicles (SVs) are energized by vacuolar H\textsuperscript{+}-dependent adenosine triphosphatases (V-ATPases) that pump protons into the vesicle lumen (1) independently of the neurotransmitter phenotype that they contain. As a result, the vesicle interior acidifies, resulting in a pH gradient (\(\Delta p\text{H}\)) and an inside positive membrane potential (\(\Delta \psi\)) that both contribute to the free energy of the gradient (\(\Delta G\)).

Across the vesicle membrane, shifting the balance between \(\Delta \psi\) and \(\Delta \rho\text{H}\) has profound influence on the uptake kinetics of different neurotransmitters; in vitro, uptake of negatively charged glutamate is maximal when \(\Delta \rho\text{H}\) dominates. In contrast, uptake of positively charged monoamines requires mainly \(\Delta \rho\text{H}\), whereas neutral \(\gamma\)-aminobutyric acid (GABA) uses both components of \(\Delta G\) (2). Because only a few hundred protons need to be translocated to saturate \(\Delta \rho\text{H}\), other ions must compensate for the transport of the estimated 2000 to 5000 transmitter molecules (2, 4). However, it has been surprisingly difficult to unravel such compensating ion fluxes and the responsible channels and/or transporters. In particular, the transport mechanism for GABA has remained enigmatic, with both GABA/H\textsuperscript{+} exchange and GABA/C\textsuperscript{+} cotransport having been proposed (5).

Mechanistic studies on vesicular transporters are generally carried out by using highly purified

REFERENCES AND NOTES


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SUPPLEMENTARY MATERIALS

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Fig. S1 to S12

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SVs or, more recently, purified transporters that are reconstituted in artificial vesicles (6–8). Although our present knowledge of transport mechanisms is largely based on such approaches, only average properties can be studied. In contrast, almost no information is available about the properties of single SVs such as kinetics, heterogeneity, or properties specific for different neurotransmitter phenotypes. To overcome these limitations, we developed an approach to measure both ∆pH and ∆w in single SVs whose neurotransmitter phenotype is subsequently identified. SVs purified from transgenic mice expressing super-ecliptic pHluorin in the vesicular lumen (spH-SVs) (9) were imaged by using total internal reflection fluorescence microscopy in order to accurately measure luminal pH changes above pH 6, as described previously (Fig. 1, A and B, and fig. S5A) (10). We combined this approach with a potentiometric assay by labeling SVs with the voltage-sensitive dye VF2.1Cl (11) to quantitatively measure ∆w across the lipid bilayer of single SVs (Fig. 1, C and D, and fig. S5C). After measuring ∆pH or ∆w, we performed on-stage labeling with antibodies against vesicular GABA (VGAT) or vesicular glutamate transporter 1 (VGLUT1) to distinguish between GABA-containing (GABAergic) and glutamatergic SVs unequivocally (Fig. 1, E and F, and fig. S4).

At all tested adenosine 5′-triphosphate (ATP) concentrations (0.6 to 3 mM), significant differences in ∆H+ were observed between glutamatergic and GABAergic SVs in the same population (significance in all experiments was determined with Student’s two-tailed t test). In the absence of monovalent ions, ∆H+ generated by the V-ATPase across the membrane of glutamatergic SVs was on average greater by 17.79 ± 6.9 (SD) mV [11.99 ± 5.2 mV larger ∆w and 0.1 ± 0.03 larger ∆pH (12)] than that of GABAergic SVs (Fig. 2, A and B). Three parameters may account for these differences in ∆H+: the rate of proton pumping; the amount of free protons in the lumen, which depends on the luminal buffering capacity (β); and the rate of proton efflux (κ). We excluded the first parameter because the rate of proton pumping was not different between glutamatergic and GABAergic SVs (fig. S6A). Next, we measured β using a modified ammonium pulse technique (fig. S7) (14). Although β increased as the lumen of vesicles acidified, there was no significant difference in β between both SV populations (fig. S8). Last, we measured the proton efflux rate in these vesicles as described previously (fig. S9) (10, 12). Significantly faster proton efflux was measured in GABAergic as compared with glutamatergic SVs (Fig. 2C). This indicates higher proton permeability (P0,H+) in these SVs (P0,H+ = 15.2 ± 10−3 and 13.5 ± 10−3 cm s−1 in GABAergic and glutamatergic SVs, respectively), thus accounting for the reduced ∆H+ in GABAergic SVs. To exclude that the higher proton efflux rate is simply caused by nonspecific leakage owing to an increased surface area, we measured the diameter of SVs with electron microscopy, using immunogold labeling to distinguish between vesicle types (fig. S10), and observed no difference between these vesicle types.

Fig. 1. ∆H+, measured at the single-vesicle level. (A) Averaged spH time-trace in response to 5-(N-Ethyl-N-isopropyl)amiloride (NEP)-ATP uncaging by an ultraviolet flash (I2). (B) Representative image of spH-SVs. (C) Averaged VF2.1Cl time-trace in response to ATP addition. (D) Representative image of VF2.1Cl-labeled SVs. (E and F) Representative spH-SVs immunolabeled for (E) VGAT and (F) VGLUT1. Dashed circles in (B) and (D) indicate detected SVs. Scale bars, 1 μm. In (A) and (C), the traces in the absence of ATP show the photobleaching of the probes, and error bars represent SEM from more than 500 single SVs.

Fig. 2. VGAT functions as a GABA/H+ antiporter. (A) Luminal pH and (B) ∆w of acidified glutamatergic and GABAergic SVs (P = 2.7 × 10−3 and 0.03, respectively). The variations in luminal pH are partially due to different ATP concentrations in some of the experiments (0.6 to 3 mM). Two circles connected via a dashed line represent one experiment. (C) Proton efflux time constant of glutamatergic versus GABAergic SVs (P = 0.03). (D) Correlation between luminal pH of acidified GABAergic SVs with intensity of antibody against VGAT (P = 0.011). (E) and (F) Proton efflux time constant of (E) GABAergic and (F) glutamatergic SVs in the presence and absence of GABA (P = 7.7 × 10−3 in (E)). Data in (A) and (B) represent mean ± SEM from on average 450 glutamatergic and 160 GABAergic SVs per experiment; in (C), (E), and (F), data represent mean ± SEM from on average 50 single SVs compiled from independent experimental replicates with a coefficient of determination (R²) > 0.7; and in (D), data represent mean ± SD of seven independent experiments. n.s., not significant (P > 0.05).
GLUTAMERTIC AND GABAERTIC SVs

TEA-Cl in the bath solution in induced by addition of 20 mM ethylammonium chloride (TEA-Cl) to acidified glutamertic SVs in the absence of 5 mM tetrathyammonium chloride (TEA-Cl) on GABAergic (red trace) but not in glutamertic (black trace) SVs. Error bars represent SEM of 50 and 392 SVs in red and black traces, respectively. (B) Changes in ΔΨ induced by addition of GABA to acidified SVs in the absence and presence of 5 mM tetrathyammonium chloride (TEA-Cl) in the bath solution. (C) ΔΨ induced by addition of 20 mM TEA-Cl to glutamertic and GABAergic SVs (P = 0.006). (D) ΔpH induced by including TEA-Cl in the bath solution in glutamertic and GABAergic SVs (P = 0.01). (E) Proton pumping rate constant of glutamertic [1.52 ± 0.15 (SEM) s⁻¹] and GABAergic [1.18 ± 0.14 (SEM) s⁻¹] with 2.4 mM ATP in the presence of Cl⁻ (P = 0.045). Data in (B), (C), and (D) represent mean ± SD of three to five independent experiments, and in (E), data represent mean ± SEM from on average 60 single SVs compiled from independent experimental replicates. n.s., not significant (P > 0.05).

Fig. 4. Cation/H⁺ exchange is more pronounced in glutamertic SVs. (A) Averaged fluorescence trace of SVs in response to 1 mM ATP and 30 mM K-glucamine (black trace) or 10 mM Na-glucamine (red trace). (B) ΔΨ induced by addition of 30 mM K-glucamine or 10 Na-glucamine to acidified SVs. (C) ΔpH induced by K-glucamine or Na-glucamine in glutamertic and GABAergic SVs (P = 0.009 for K⁺ effect). (D) Inhibition of Na⁺ but not K⁺ induced ΔpH by EIPA (P = 0.002 for Na⁺ effect). (E) Changes in ΔΨ induced by addition of 10 mM N-methyl-D-glucamine (NMDG)–glutamate and K-glutamate to acidified SVs (P = 0.02). Data in (A) represent mean ± SEM from on average 600 single SVs, and in (B) to (E), data are mean ± SD of three to five independent experiments.

Fig. 3. VGAT is not a GABA/Cl⁻ cotransporter. (A) Averaged VF2.1.C time-trace in response to addition of 3 mM ATP and 10 mM GABA, indicating changes in ΔΨ associated with GABA uptake in GABAergic (red trace) but not in glutamertic (black trace) SVs. Error bars represent SEM of 50 and 392 SVs in red and black traces, respectively. (B) Differences in ΔΨ induced by addition of GABA to acidified SVs in the absence and presence of 5 mM tetrathyammonium chloride (TEA-Cl) in the bath solution. (C) ΔΨ induced by addition of 20 mM TEA-Cl to acidified glutamertic and GABAergic SVs (P = 0.006). (D) ΔpH induced by including TEA-Cl in the bath solution in glutamertic and GABAergic SVs (P = 0.01). (E) Proton pumping rate constant of glutamertic [1.52 ± 0.15 (SEM) s⁻¹] and GABAergic [1.18 ± 0.14 (SEM) s⁻¹] SVs with 2.4 mM ATP in the presence of Cl⁻ (P = 0.045). Data in (B), (C), and (D) represent mean ± SD of three to five independent experiments, and in (E), data represent mean ± SEM from on average 60 single SVs compiled from independent experimental replicates. n.s., not significant (P > 0.05).

populations. These data strongly suggest that a protein specific for GABAergic SV is responsible for the increased proton efflux rate. Because only the vesicular transporters are exclusively present in each vesicle population (25), we hypothesized that VGAT contributes to proton efflux in GABAergic SVs. Indeed, in each acidification measurement, the luminal pH of acidified GABAergic SVs correlated with the immunolabeling intensity for VGAT, indicating that SVs with greater VGAT copy numbers had greater proton permeability (Fig. 2D).

As discussed above, the transport mechanism of VGAT is controversial, with both GABA/H⁺ antiport (16–19) and GABA/Cl⁻ cotransport (8) having been proposed. In the former case, proton efflux of GABAergic SVs should be enhanced when GABA is added. Indeed, we observed significantly accelerated proton efflux from GABAergic vesicles (Fig. 2E) in the presence of 10 mM GABA, whereas that of glutamertic SVs remained unchanged (Fig. 2F), clearly indicating that VGAT does in fact function as a GABA/H⁺ exchanger. The same result was obtained with 20 mM glycine, which is another substrate of VGAT (Fig. S11A and B). However, glutamate had no significant effect on proton efflux rate in any of the vesicle populations (Fig. S12).

The experiments described above were carried out in the absence of Cl⁻. To test whether Cl⁻ contributes to GABA transport, we characterized the effects of GABA and Cl⁻ on ΔΨ. When vesicles were allowed to acidify, addition of GABA partially dissipated ΔΨ exclusively in GABAergic SVs (Fig. 3A), confirming that GABA (no net charge at neutral pH) is exchanged for protons. When Cl⁻ was included, only a slightly higher reduction of ΔΨ was observed (Fig. 3B), ruling out any prominent GABA-coupled transport of Cl⁻ by VGAT (8). Thus, the stimulatory effect of Cl⁻ on GABA uptake observed previously (5, 18, 19) is probably due to a shift from ΔΨ to ΔpH. Indeed, addition of 20 mM Cl⁻ to acidified vesicles caused a shift in the ΔΨ tension balance, dissipating the membrane potential while enhancing luminal acidification (Fig. 3, C and D, and fig. S14). Cl⁻ influx, presumably through the vesicular Cl⁻/H⁺ exchanger 3 (CIC3) (20) or VGLUT2 (21), effectively shuts the membrane potential, allowing the V-ATPase to drive more protons into the lumen, which in turn are exchanged for GABA by VGAT. Addition of Cl⁻ resulted in significantly larger dissipation of ΔΨ and greater acidification in glutamertic rather than in GABAergic SVs (Fig. 3C and D). Moreover, Cl⁻ selectively enhanced the rate of proton pumping in glutamertic SVs (Fig. 3E). Because CIC3 is present in both vesicle types in comparable quantities (25), our data indicate that VGLUT directly contributes to Cl⁻ transport, which is in agreement with previous reports (6, 7).

Last, we examined whether Na⁺ and K⁺ exert different effects on the two vesicle populations. Both ions convert ΔΨ to ΔpH via cation/H⁺ exchange mechanisms (22) but involve different pathways: Na⁺/H⁺ exchange is mediated by the Na⁺/H⁺ exchanger NHE6, whereas K⁺/H⁺ exchange is proposed to be mediated by VGLUT (6). Our single-vesicle assay allowed for discriminating these two exchange activities in single vesicles. Indeed, addition of each cation to acidified vesicles significantly alkalized the luminal pH (Fig. 4A and fig. S14A) and slightly increased the
membrane potential (Fig. 4B), but with notable differences between the two vesicle populations: Less K⁺-induced alkalinization was observed in GABAergic SVs, whereas Na⁺-induced similar alkalinization levels in both vesicle populations (Fig. 4C). When the experiments were repeated in the presence of the NHE inhibitor ethyl-isopropyl amiloride (EIPA), Na⁺-induced alkalinization was significantly blocked, whereas K⁺-induced alkalinization remained unchanged (Fig. 4D). The greater effect of K⁺ in glutamatergic SVs may be due to the presence of GABAergic neurons (21). It is also possible that VGAT contributes to the measured K⁺/H⁺ exchange but with a different stoichiometry than that of VGLUT. Both the NHE-mediated Na⁺/H⁺ and VGLUT-mediated K⁺/H⁺ exchange convert ΔpH to Δψ, counteracting the effect of Cl⁻ on Δψ₁. Moreover, we observed that dissipation of Δψ by glutamate uptake (fig. S15) was significantly mitigated by the presence of K⁺ (Fig. 4E). This further emphasizes the crucial role of VGLUT-mediated K⁺/H⁺ exchange as a charge-compensating mechanism that helps to sustain Δψ and enhances glutamate uptake (6).

Through the quantitative characterization of Δψ₁, in single vesicles, we report here that not only the pH gradient of Δψ₁ (20) but also Δψ is generated across the membrane of SVs within 1 to 2 s. Moreover, our data clearly demonstrate that VGAT is not a GABA/Cl⁻ cotransporter (8) but functions as a GABA/H⁺ antiporter. Surprisingly, our data suggest that VGAT is leaky to protons in the absence of substrate, raising the question of whether the presence of GABA prevents nonspecific proton leakage, replacing it with a strict GABA/H⁺ antiport. In addition, ion fluxes through VGLUT can shift the balance of the components of Δψ₁, toward greater Δψ and ensure charge and osmolarity compensation during neurotransmitter loading. Thus, neurotransmitter transporters are critical regulators of Δψ₁, which adds another level of complexity to their contribution to maintenance of fidelity in synaptic transmission.

REFERENCES AND NOTES

12. Materials and methods are available as supplementary materials on Science Online.

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Single-vesicle imaging reveals different transport mechanisms between glutamatergic and GABAergic vesicles
Zohreh Farsi, Julia Preobraschenski, Geert van den Bogaart, Dietmar Riedel, Reinhard Jahn and Andrew Woehler

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**Neurotransmitter uptake one vesicle at a time**

Despite opposing ionic gradients, synaptic vesicles are able to accumulate neurotransmitters. To resolve the mystery of how this happens, Farsi et al. made parallel measurements of pH gradients and membrane potential at the single synaptic vesicle level. Glutamatergic and GABAergic vesicles had different uptake mechanisms, revealing insights into the energetic and ionic coupling of vesicular neurotransmitter transport.

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