

Differential Sorting of the Vesicular Glutamate Transporter 1 into a Defined Vesicular Pool Is Regulated by Light Signaling Involving the Clock Gene *Period2**

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Synaptic strength depends on the amount of neurotransmitter stored in synaptic vesicles. The vesicular transmitter content has recently been shown to be directly dependent on the expression levels of vesicular neurotransmitter transporters indicating that the transport capacity of synaptic vesicles is a critical determinant for synaptic efficacy. Using synaptic vesicles prepared from whole brain at different times of the day we now show that the amount of vesicular glutamate transporter (VGLUT) 1 undergoes strong diurnal cycling. VGLUT1 protein levels are high before the start of the light period, decline at noon, increase again before start of the dark period, and decline again at midnight. Mice kept in complete darkness showed within a 24-h period only a single peak of VGLUT1 expression in the middle of the rest phase. In contrast, mice lacking the period gene *Period 2*, a core component of the circadian clock, did not show any light-cycle-dependent changes of VGLUT1 levels. No other of several synaptic vesicle proteins examined underwent circadian cycling. Circadian cycling of VGLUT1 was not seen when analyzing homogenate or synaptosomes, the starting fraction for vesicle preparation. Circadian cycling of VGLUT1 was also not reflected at the mRNA level. We conclude that nerve terminals are endowed with mechanisms that regulate quantal size by changing the copy number of transporters in synaptic vesicles. A reduced amount of VGLUT1 per vesicle is probably achieved by means of selective sorting controlled by clock genes.

All major biological functions of mammals follow a pattern of daily rhythms depending on the rotation of the earth and the repeated rising and setting of the sun. In the absence of environmental cues, *i.e.* the natural light-dark cycle, endogenous rhythms, termed circadian rhythms, display a periodicity close to 24 h, reflecting the intrinsic biological clock. Circadian rhythms in different tissues including brain are orchestrated by a master clock located in the suprachiasmatic nucleus. This clock synchronizes physiological and biochemical pathways thereby allowing the organism to anticipate daily requests and adapt to environmental changes. In the last years, genetic analyses revealed two transcription factors CLOCK and BMAL1 that induce transcription of *Period* (*Per1*, *Per2*, and *Per3*) and *Cryptochrome* (*Cry1* and *Cry2*) genes. The *Per* and *Cry* gene prod-

ucts in turn are thought to inhibit the activity of CLOCK and BMAL1 and thereby constitute a negative feedback loop (1, 2). This molecular principle appears to exist in almost every cell.

Among the three *Per* genes *Per2* seems to play a critical role in adaptation of the clock to the environmental light-dark cycle. Notably *Per2^{Brdm1}* mutant mice that carry a deletion in the PAS domain of the *Per2* protein display impaired clock resetting in response to light (3) and an increase in alcohol consumption (4). These findings are most likely related to a hyperglutamatergic state in these mice due to a reduction in the expression of the glutamate transporter EAAT1 (4). Although astrocytic plasma membrane glutamate transporters like EAAT1 are responsible for clearing the synaptic cleft from presynaptically released glutamate, vesicular transporters concentrate glutamate in synaptic vesicles of the presynapse, thus filling them for the next round of exocytosis. Two isoforms of vesicular glutamate transporters (VGLUT³)1 and -2) have been identified in glutamatergic neurons and a third occurs as a cotransmitter transporter in other types of neurons (Ref. 5 and references therein). Studies of VGLUT1^{-/-} mice revealed that the amount of transporter per vesicle is crucial for the amount of glutamate loaded and that VGLUT1 and VGLUT2 target to functionally distinct synaptic release sites (6, 7).

Here we analyzed the protein patterns of VGLUT1 in synaptic vesicles (SV) over 24 h in wild type and *Per2^{Brdm1}* mutants entrained under a light-dark cycle or in complete darkness. The data suggest a novel sorting mechanism by which the presynaptic terminal may regulate its transmitter output.

EXPERIMENTAL PROCEDURES

Animals—*Per2^{Brdm1}* mice used in this study were characterized previously (8). The wild type and *Per2^{Brdm1}* animals used represent littermates derived from intercrosses between heterozygous *Per2^{Brdm1}* mice on a 129SvEvBrd/C57/BL/6-Tyrc-Brd background. Mice were kept under either an uninterrupted 12 h light/12 h dark or dark/dark cycle for at least 7 or 3 days, respectively, before the start of the experiment (9).

Antibodies—The following antibodies were generous gifts from R. Jahn, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany: mouse monoclonal antibodies against synaptobrevin II (clone 69.1, Ref. 10), synaptophysin (clone 7.2, Ref. 11), and VGLUT1 and -2. Both VGLUT1 antibodies are isoform-specific with no cross-reactivity against VGLUT3 (12, 13). Monoclonal antibodies against synaptotagmin, Rab3A, and synapsin were purchased from Synaptic Systems, Göttingen. Polyclonal rabbit antibodies against VGLUT1 and the 116-kDa subunit of the vacuolar proton pump were from Synaptic Systems,

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³ The abbreviations used are: VGLUT, vesicular glutamate transporter; SV, synaptic vesicle; ZT, Zeitgeber time; CT, circadian time; PIPES, 1,4-piperazinediethanesulfonic acid; LD, light/dark; DD, dark/dark.

Göttingen, Germany and against actin from Sigma, respectively. Secondary antibodies for Western blot detection, horse anti-mouse and goat anti-rabbit conjugated with horseradish peroxidase were purchased from Vector Laboratories, Burlingame, CA.

Synaptosomes and Synaptic Vesicles—Isolated nerve terminals (synaptosomes) were prepared at 4 °C from adult mouse whole brains in the presence of protease inhibitors. SV (LP2 fraction) were prepared from mouse brains following the procedure described (10, 14). Mice were sacrificed at the given time points either in the light/dark cycle (Zeitgeber time, ZT) or the dark/dark cycle (circadian time, CT).

Generally, wild type and *Per2^{Brdm1}* mutants were analyzed in parallel. Protein determination was performed from the individual membrane fractions and equal amounts of protein were loaded for SDS-PAGE.

For each set of experiments membrane fractions were run in parallel, and proteins were transferred to nitrocellulose and further processed for immunodetection using the ECL detection system. In some experiments especially when homogenates were analyzed, Triton X-114 phase partitioning was performed to extract membrane proteins. Briefly, 0.5 mg of protein was extracted with partitioning buffer (10 mM Tris-HCl, 150 mM NaCl, pH 7.4) containing 1% Triton X-114 for 15 min on ice followed by an incubation for 5 min at 37 °C until the sample turned cloudy. The sample was centrifuged at 5000 × *g* for 2 min resulting in a lower detergent phase and an upper aqueous phase. The upper aqueous phase was extracted once again with 1% Triton X-114. The detergent phases from both the extractions were pooled, and protein was precipitated by the conventional methanol/chloroform (1:1) precipitation method. The final protein pellet was air-dried thoroughly before dissolving in Laemmli buffer (15).

ECL-processed films were scanned by video imaging, and protein bands were densitometrically quantified using the SCAN PACK 3.0 program. It was ensured that the signals are in the linear range of the ECL detection system. Quantification was performed as described (16) along with statistical analysis using the paired Student's *t* test.

Glutamate Uptake—Glutamate uptake was performed with vesicles prepared and frozen at the indicated time points. A comparison between frozen and freshly prepared vesicles ensured that there was no difference in the overall glutamate uptake. Thawed vesicles were suspended in KGC buffer (150 mM K⁺-gluconate, 20 mM PIPES, 4 mM EGTA, 2 mM MgCl₂, pH 7.0, KOH), and 25 μl of this suspension (corresponding to 30–60 μg of protein) were added to individual tubes containing 25 μl of KGC supplemented with 4 mM ATP (2 mM final ATP concentration in KGC/ATP buffer) and with 158.4 μM glutamate and 1.6 μM [³H]glutamate (L-[G-³H]glutamic acid, specific activity 1220 Bq/mmol, Amersham Biosciences). Uptake was performed for 10 min at 36 °C and stopped by adding 500 μl of ice-cold KGC/ATP buffer. Samples were spun down (10 min, 460,000 × *g*) and washed once using KGC/ATP buffer. The pellets were lysed with 0.4% Triton X-100 to determine radioactivity by scintillation counting and protein content by the BCA method. Nonspecific binding was performed in the presence of 5 μM nigericin and 20 μM valinomycin and reached values between 0.05 and 0.12 nmol/mg of protein. Values were expressed as nmol/mg of protein and corrected for nonspecific binding (17).

In Situ Hybridization—Mice were sacrificed by cervical dislocation under ambient light conditions at ZT6 and ZT12 and under a 15 W safety red light at ZT18 and ZT24. Brains were quickly dissected out and fixed in 4% paraformaldehyde in phosphate-buffered saline, pH 7.4, at 4 °C for 12–18 h. Tissues were then dehydrated with ethanol (30, 50, 70, and 100%, 3 h each at 4 °C) and transferred to xylene at room temperature. Xylene was changed once before being replaced by 50% xylene/50% paraffin. Then tissue was placed in paraffin, which was exchanged

three times at 60 °C before pouring it in embedding boats. Brain sections (7 μm), were cut using a microtome (R. Jung, Hamburg, Germany) and stored at room temperature until further use.

The probes for *VGLUT1* were made from cDNA corresponding to nucleotides 801–1380 (GenBankTM accession number NM_053859) and for *VGLUT2* from nucleotides 1579–2238 (GenBankTM accession number NM_053427). ³⁵S rUTP-labeled riboprobe synthesis and hybridization steps were performed as described (18).

RESULTS

Oscillation of the Amount of VGLUT1 in SV under Light/Dark and Dark/Dark Conditions—A variety of vesicular integral membrane and associated proteins including the vesicular transmitter transporters VGLUT1 and VGLUT2 were analyzed in SV fractions obtained from whole brain of wild type and *Per2^{Brdm1}* mice entrained in a 12 h light/12 h dark (LD 12:12) cycle at the indicated time points (Fig. 1). The time points ZT refer to ZT24/0 at 6:00 a.m. before the light was switched on, ZT6 at noon, ZT12 at 6:00 p.m., dawn, before the light was switched off, and ZT18 at 12:00 a.m. in the middle of the night. The amount of VGLUT1 in the SV fractions varied over the LD cycle being high at ZT24/0 and ZT12 before the light was switched on or off, respectively, and low in either the middle of the day or the night (Fig. 1A). Relative amounts of vesicular proteins based on ratios to the amount of synaptophysin as an abundant integral vesicular membrane protein and actin as a further internal control were taken. Quantification over 4–6 independent experiments revealed a significant change in the amount of VGLUT1 in the SV fraction dependent on the time of day. VGLUT1 to synaptophysin ratios were high just before the beginning of the light phase (ZT24/0) and the dark phase (ZT12), respectively, and were reduced to about half of these amounts in the middle of the day (ZT12) and the night (ZT18). VGLUT2 also varied slightly, but these variations were less pronounced (Fig. 1A). The protein pattern of VGLUT1 was altered in SV obtained from *Per2^{Brdm1}* mice with a more even expression over the day and a variable drop at midnight (Fig. 1B). The amount of other vesicular proteins like synaptophysin, synaptotagmin, and synaptobrevin or the vesicle-associated proteins Rab3 and synapsin and their ratios to synaptophysin varied neither in wild type nor in *Per2^{Brdm1}* mice (Fig. 1). When mice were entrained to complete darkness (DD) the pattern obtained for the amount of VGLUT1 to synaptophysin ratio in the SV fractions changed remarkably. The maximum shifted to CT6 (Fig. 2A) in wild type mice with a double amount of the VGLUT1 to synaptophysin ratio compared with CT18. The observed variation was confirmed with a different polyclonal antibody (Fig. 2A, antibodies *m* and *p*). This pattern of the VGLUT1 to synaptophysin ratio was not altered in the *Per2^{Brdm1}* mutants and resembles the pattern observed under LD conditions in *Per2^{Brdm1}* mutants (compare Fig. 2B with 1B), whereas the amounts of other vesicular proteins were identical irrespective of the time of day (Fig. 2). Because *Per2^{Brdm1}* mutants get arrhythmic in complete darkness after 4 days or more, animals were sacrificed at the third day of the changed entrainment. At this time point rhythmicity still persists in the mutants, allowing for a documentation of their respective circadian time (3).

VGLUT1 Protein and mRNA Levels in Various Brain Fractions and in Brain Sections—The data indicate that the amount of VGLUT1 varies in the SV fraction dependent on the time of day. These variations may reflect circadian-dependent protein synthesis or degradation. Alternatively a selective sorting out of VGLUT1 from the SV to another membrane pool by membrane traffic could be considered. To discriminate between these two possibilities we analyzed the initial homogenate and the synaptosomes, which represent the starting material for the prepa-

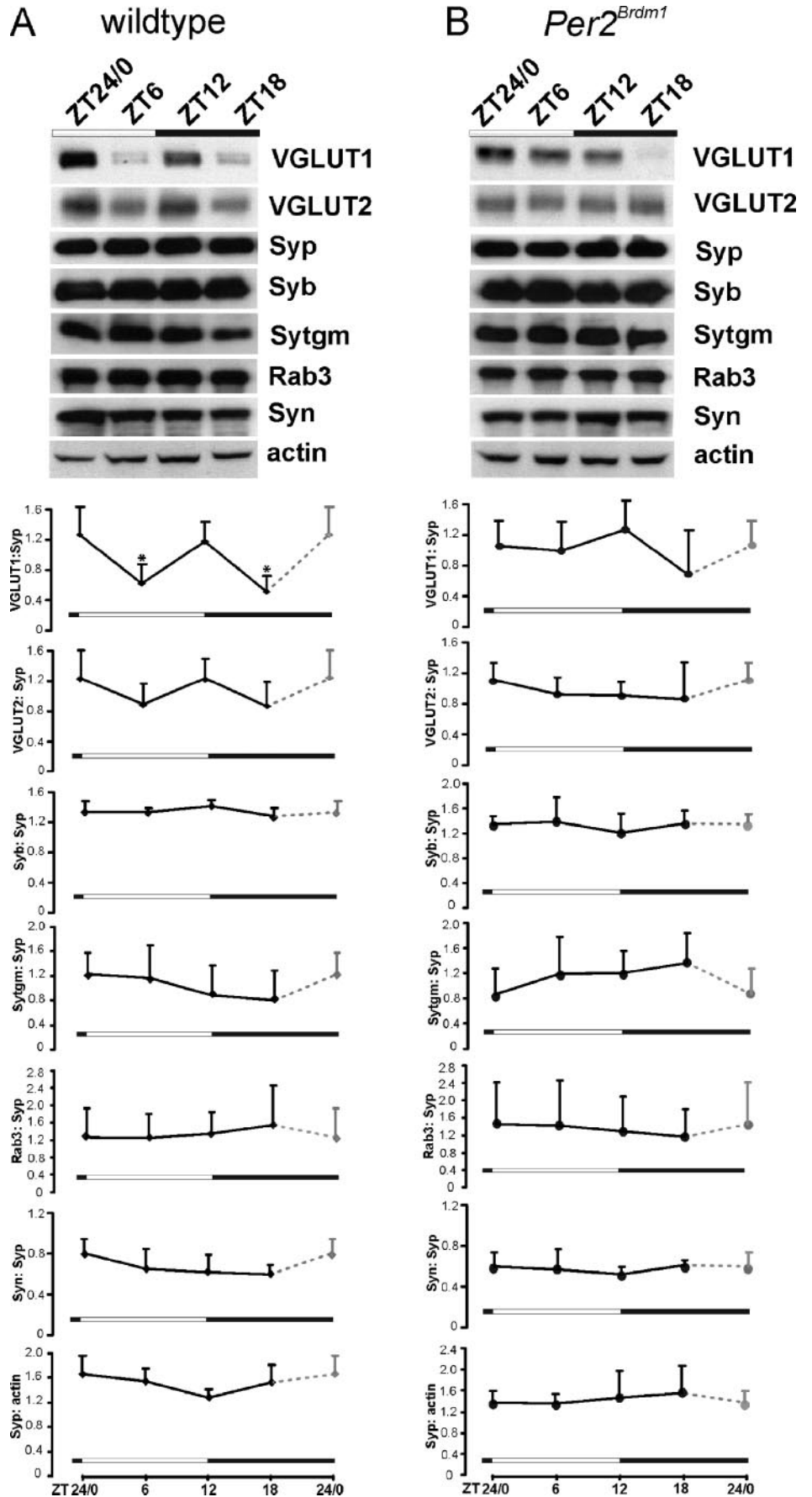


FIGURE 1. Oscillation of VGLUT1 amount in SV under light/dark condition. SVs were prepared at the indicated time points either from wild type (A) or *Per2^{Brdm1}* (B) mice. ZT24/0 represents dawn at 6:00 a.m., before the light was switched on (refers to ZT24), ZT6 represents noon at 12:00 p.m., ZT12 is dusk at 6:00 p.m. (before the light was switched off), and ZT18 is night at 12:00 a.m. The light/dark condition is also reflected by the white/black bar heading the blot documentation and below each individual graph. The vesicular membrane fractions were analyzed for the proteins indicated. Quantification was performed by calculating the ratio of synaptophysin to VGLUT1, VGLUT2, or the other SV proteins indicated. The amount of synaptophysin was calibrated against the amount of actin. Values are obtained in three to five different sets of experiments. Stars denote significance ($p < 0.05$) according to Student's *t* test between time points ZT24/0 or ZT12 and ZT6 or ZT18, respectively. *Syp*, synaptophysin; *Syb*, synaptobrevin; *Sytgm*, synaptotagmin; *Syn*, synapsin.

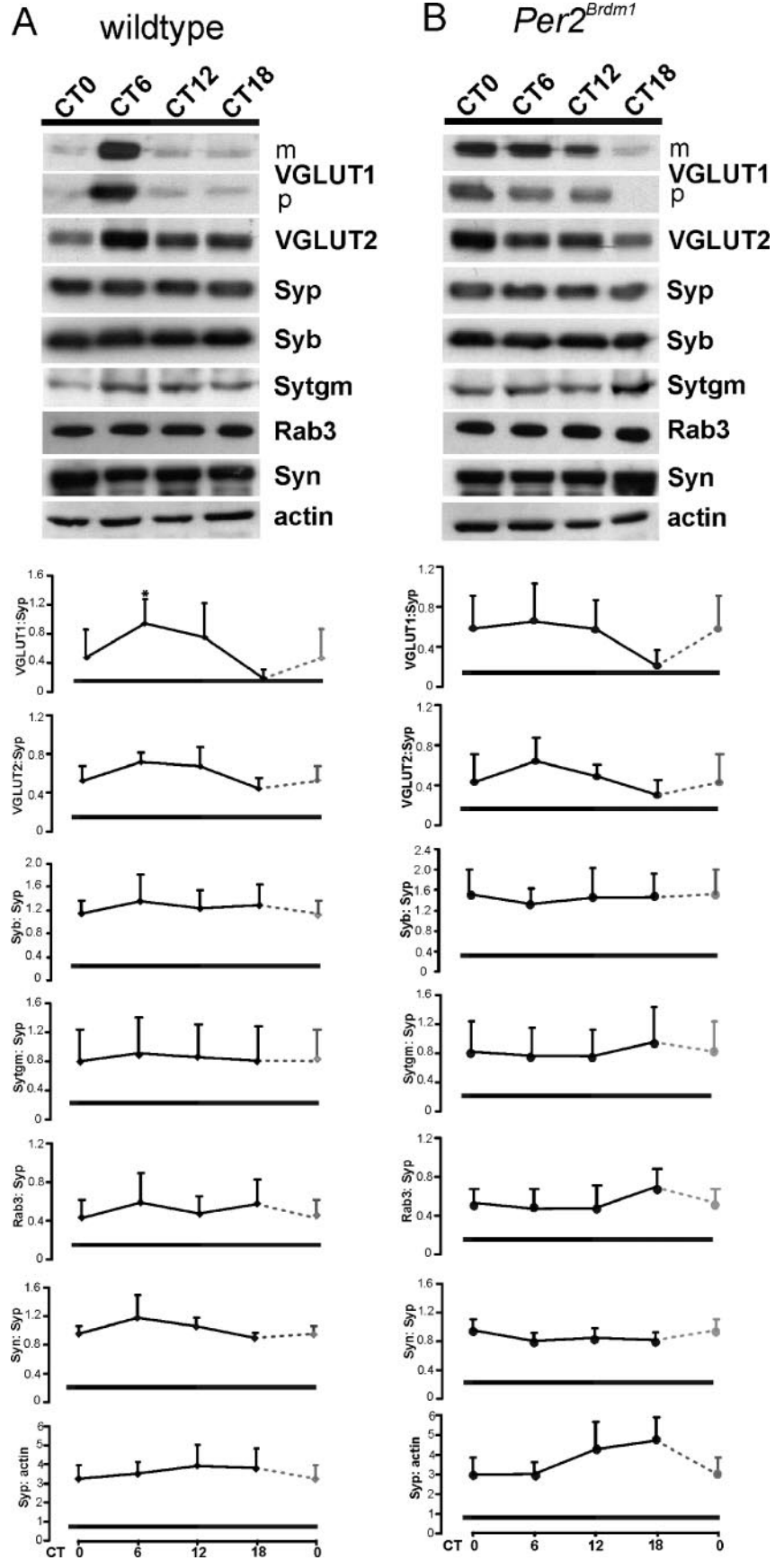


FIGURE 2. **Oscillation of VGLUT1 amount in SV under dark/dark condition.** SVs were prepared at the indicated time points either from wild type (A) or *Per2^{Brdm1}* (B) mice. The dark/dark condition is also reflected by the *black bar* heading the blot documentation and below each individual graph. The vesicular membrane fractions were analyzed for the proteins indicated. Quantification was performed by calculating the ratio of synaptophysin to VGLUT1, VGLUT2, or the other SV proteins indicated. The amount of synaptophysin was calibrated against the amount of actin. Values are obtained in three to five different sets of experiments. Stars denote significance ($p < 0.05$) according to Student's *t* test between time points CT6 and CT18.

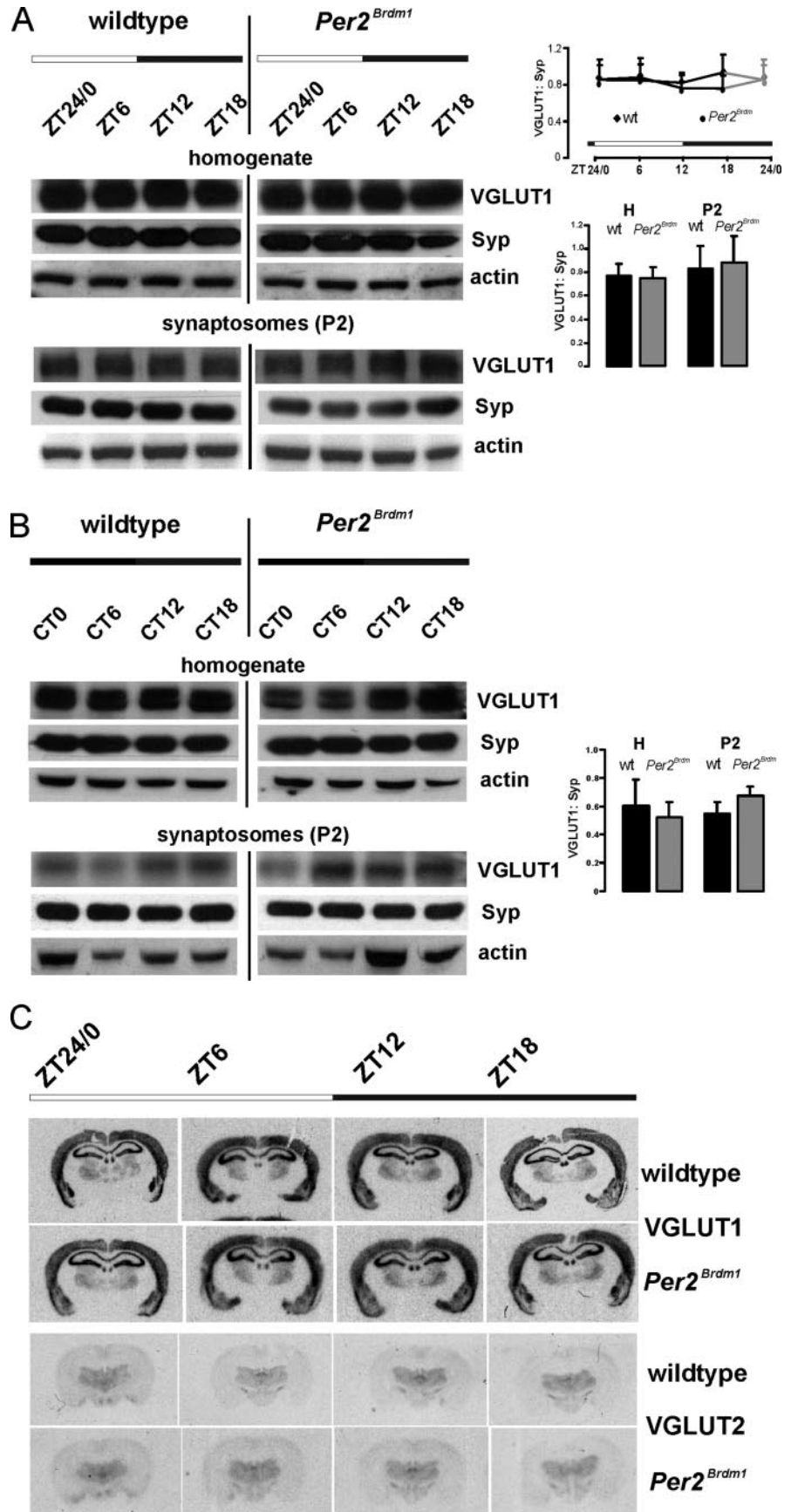


FIGURE 3. VGLUT1 at the protein and mRNA level in various brain fractions and in whole brain sections. Homogenate and synaptosomes representing the starting material for SV were analyzed in parallel from wild type or *Per2^{Brdm1}* mice entrained either under LD (A, white/black bar) or DD (B, black bar) conditions. Blots were stained with the antibodies indicated. The ratio of VGLUT1 to synaptophysin in synaptosomes (P2) (taken from three different sets) was quantified at the different ZT time points as in Fig. 1 (upper graph in A). In addition the average ratio of VGLUT1 to synaptophysin calculated from all ZT (lower graph in A) and CT (graph in B) time points in homogenate and P2 is shown. Note that the amounts of VGLUT1 and VGLUT2 in homogenates or synaptosomes are not changed between the different time points under either condition. Proteins from homogenate were extracted by Triton X-114 partitioning. *In situ* hybridization using VGLUT1 and VGLUT2 probes in whole brain sections (C, white/black bar) confirmed that the overall synthesis is not changed during circadian rhythm and between wild type and *Per2^{Brdm1}* mice.

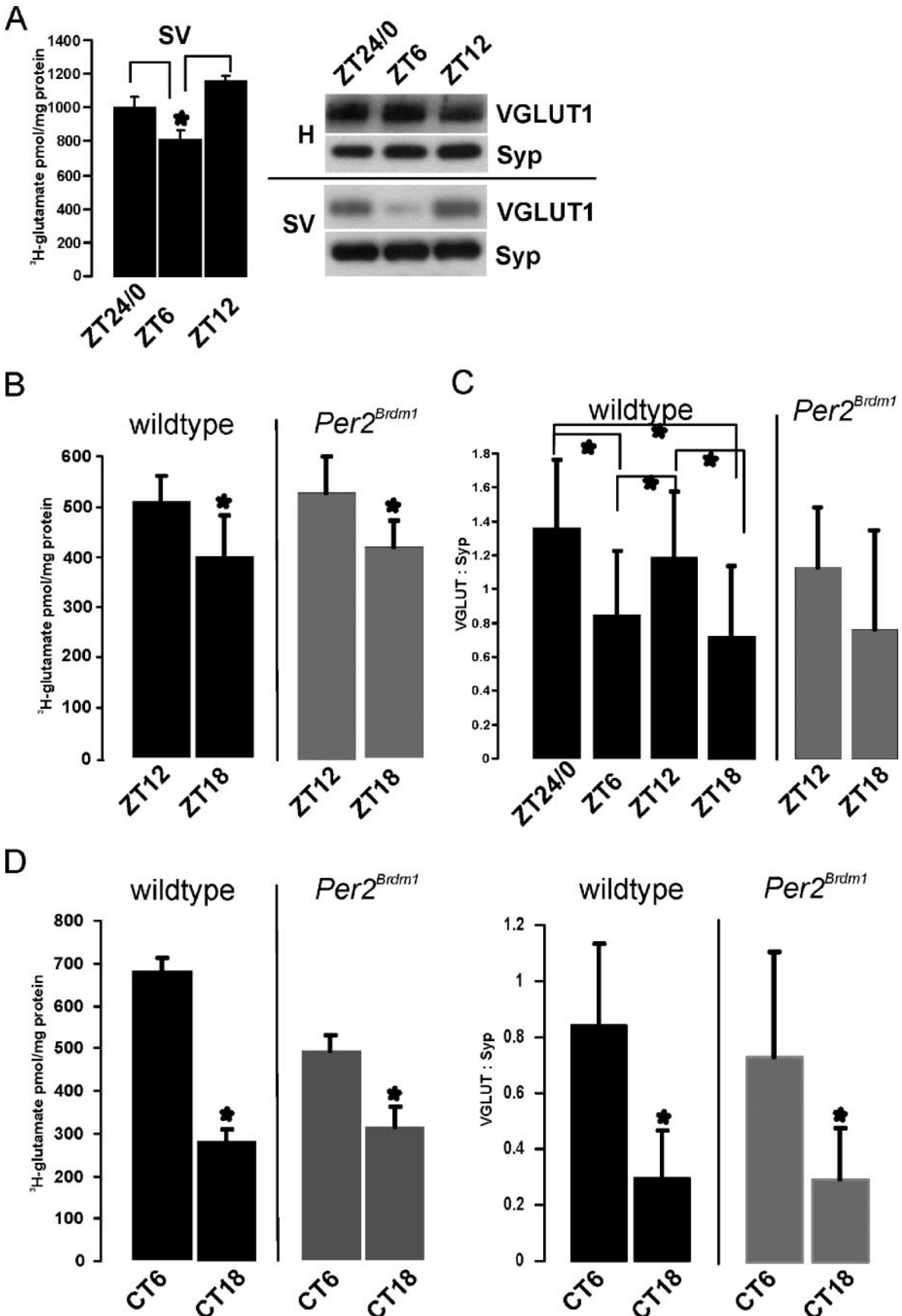


FIGURE 4. Glutamate uptake reflects the oscillation of VGLUT1. A–C, SV were prepared at the given time points of a LD cycle and analyzed for glutamate uptake or the amount of VGLUT1 and synaptophysin (Syp). The uptake is reduced at ZT6 parallel to a drop in the amount of VGLUT1 with no change in the amount of synaptophysin. In the homogenate extracted by Triton X-114 partitioning and analyzed for comparison the amounts of VGLUT1 and synaptophysin are similar at all ZT time points. Glutamate uptake was performed into

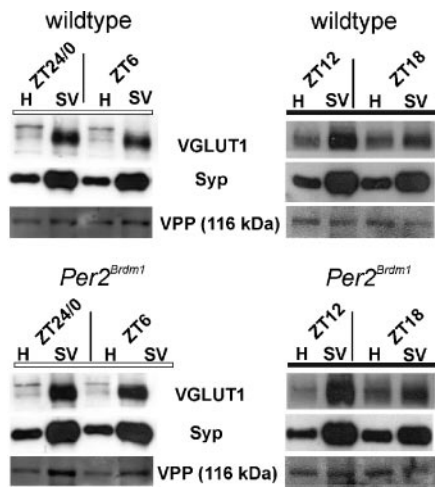


FIGURE 5. The vacuolar proton pump, synaptophysin, and VGLUT1 in homogenate and SV under LD condition. Homogenate or SVs were prepared at the given ZT time points and analyzed for the presence of VGLUT1, synaptophysin (*Syp*), and the 116-kDa subunit of vacuolar proton pump (*VPP* 116 kDa). Individual samples from two different sets with either ZT24/0 and ZT6 (light phase of a LD cycle, *white bar*) or ZT12 and ZT18 (dark phase of a DD cycle, *black bar*) are shown. All homogenate fractions were extracted by Triton X-114 partitioning. The following ratios were obtained: VGLUT1 to *Syp* in homogenate was between 1.1 and 1.0 for all conditions, for SV ZT24/0, 0.83; ZT6, 0.33; wild type and ZT24/0, 0.83; ZT6, 0.7 *Per2^{Brdm1}* mice and ZT12, 0.75; ZT 18, 0.6; wild type and ZT12, 1.3; and ZT18, 0.7 *Per2^{Brdm1}* mice. VPP to *Syp* varied between 0.45 and 0.46 for all conditions.

ration of SV. As can be seen in Fig. 3A there were no considerable variations in the ratios of VGLUT1 to synaptophysin in homogenates or synaptosomes either in the LD (Fig. 3A) or in the DD conditions (Fig. 3B). The quantification of the ratios between the amounts of VGLUT1 and synaptophysin in synaptosomes analyzed at the given ZT time points showed no oscillation at all (Fig. 3A, *upper graph*) and contrasts to the situation seen in the SV fractions (see Fig. 1A). The average VGLUT1 to synaptophysin ratios over all time points, for homogenate and synaptosomes in wild type and *Per2^{Brdm1}* mutants, respectively, again showed no apparent difference between the two genotypes (Fig. 3A). We also analyzed the VGLUT1 to synaptophysin ratios over all CT time points (DD condition) in homogenate and synaptosomes for wild type and *Per2^{Brdm1}* mutants. Comparable to the LD condition no changes between the different CT time points were observed in the DD condition in both genotypes (Fig. 3B). As a second approach we performed an *in situ* hybridization for VGLUT1 and VGLUT2 in whole brain sections obtained from brains removed at the given time points (ZT). No oscillations in the expression of VGLUT1 or VGLUT2 were observed over the 24 h of a LD cycle (Fig. 3C). In addition there was no difference in the expression of VGLUT1 and VGLUT2 in *Per2^{Brdm1}* mutants compared with wild type littermates (Fig. 3C). A direct comparison between synaptosomes obtained from three different sets of wild type and *Per2^{Brdm1}* mutants at time point ZT24/0 or CT6, the time points with the highest VGLUT1 content in SV, revealed no difference in the overall amount of proteins under the LD condition. However, the overall ratio calculated from all time points of Rab3 and to a lesser extent of VGLUT1 to synaptophysin in synaptosomes was slightly decreased in *Per2^{Brdm1}* mutants under DD conditions (data not shown).

Glutamate Uptake Reflects the Oscillation of VGLUT1—The amounts of VGLUT1 vary in wild type animals according to either light

schedule or circadian time, which suggests variations in transmitter release at different time points of the day. Vesicular filling is a reliable process and directly depends on the amount of transporter per vesicle as has been shown by overexpressing VGLUT1 (7). Generally variations in the amplitude of postsynaptic responses are taken as an indication of changes in the vesicular filling and thus on the amount of transporter per vesicle. However, other factors like the activity of plasma membrane transporters, affinity of the receptors, and the overall morphology of the synapse may contribute to and obscure changes in VGLUT amounts at the level of the vesicle (5). In addition removal of parts of the brain as necessary for electrophysiological analysis in slice preparations will probably lead to an interruption and a loss of the higher integration sustained by the suprachiasmatic nucleus. As a consequence the strict uniform entrainment for all systems will collapse, and the removed tissue may rely on its own rhythmicity no longer depending on circadian mechanisms. To overcome these restrictions we used another more direct approach by analyzing the amount of glutamate taken up in vesicular fractions prepared at the given ZT or CT. Corresponding to the amount of VGLUT1 in SV the glutamate uptake was significantly reduced at ZT6 compared with ZT24/0 and ZT12 (Fig. 4A). The parallel immunoreplica analyses of the homogenate and the SV for VGLUT1 and synaptophysin confirmed the circadian changes of VGLUT1 exclusively found in the SV fraction but not in the homogenate in wild type mice (Fig. 4A). In a second experiment SV from wild type and *Per2^{Brdm1}* mutants prepared at ZT12 and ZT18 were compared for glutamate uptake. Corresponding to the amounts of VGLUT1 at these time points glutamate uptake was reduced at ZT18 compared with ZT12 in both wild type and mutants (Fig. 4B). Because the activities of both VGLUT1 and VGLUT2 contribute to the glutamate uptake, the ratios of both VGLUT isoforms to synaptophysin were combined from the data in Fig. 1, which reflected the changes in the glutamate uptake at the various time points (Fig. 4C). Accordingly, in SV obtained from mice entrained in a DD cycle glutamate uptake was reduced by almost 60% at CT18 compared with CT6 reflecting the reduced protein levels of both VGLUT1 and VGLUT2 given as combined VGLUT to synaptophysin ratios (Fig. 4D). Together these data indicate that the circadian-specific oscillations in the amount of VGLUT1 and to a lesser extent of VGLUT2 are also reflected by the storage capacity of SV.

Generally, transmitter uptake strongly depends on the presence of the vacuolar proton pump, which is involved in the acidification of various intracellular organelles including SV. There was no difference in the ratio between the vacuolar proton pump identified by an antibody against its 116-kDa subunit and synaptophysin among time points ZT24/0, ZT 6, and ZT12, ZT18 in SV of wild type, and *Per2^{Brdm1}* mice under LD conditions. The ratios between VGLUT1 and synaptophysin analyzed for comparison revealed the expected differences between the two time points (see also Fig. 1). In addition the ratios between VGLUT1 or proton pump and synaptophysin did not vary in the homogenate fractions. As expected synaptophysin and VGLUT1 are enriched in the SV fraction compared with homogenate, whereas the enrichment in the vacuolar proton pump due to its occurrence on a variety of organelles was less pronounced (Fig. 5).

SV prepared at the time points ZT12 and ZT18 of a LD cycle from wild type and *Per2^{Brdm1}* mice. The decrease in the uptake (~25% in ZT6 and ZT 18 compared with the other time points) reflects the decrease in the amount of VGLUT in total to synaptophysin (37%) in the respective fractions from wild type and *Per2^{Brdm1}* mice. D, SV were prepared at the given time points (CT) of a DD cycle from wild type and *Per2^{Brdm1}* mice. The decrease in the uptake (~60% in wild type and 30% in *Per2^{Brdm1}* mice) reflects the decrease in the amount of VGLUT in total to synaptophysin (60% in wild type and 50% in *Per2^{Brdm1}* mice) in this fraction in both wild type and mutant mice. Quantification of the VGLUT to synaptophysin ratios at the indicated time points include values combined from individual VGLUT1 and VGLUT2 to synaptophysin ratios from Figs. 1 and 2. *Stars* denote significance according to Student's *t* test with $p < 0.05$.

DISCUSSION

In the present work we showed that the amount of VGLUT1 in a given vesicular fraction oscillates during a 24-h LD cycle. This oscillation depends on light and requires the presence of a functional *Per2* gene. The data have two implications. They describe for the first time a link between the circadian system, light input, and synaptic efficiency. In addition and equally important they show that the presynaptic terminal can change the equipment of integral membrane proteins of SV by a sorting mechanism in an activity-dependent manner, in this case based on circadian rhythmicity and light input.

Light-induced information is transduced from the retina via the retinohypothalamic tract to the suprachiasmatic nucleus eliciting glutamate release at the synapse of the retinohypothalamic tract. This signal is the major cue to induce resetting of clock phase in mammals. Interestingly, *Per2^{Brdm1}* mutant mice have a defect in delaying clock phase, which is thought to be because of an altered glutamatergic signaling in these animals (4). Our data indicate that the majority of VGLUT1-type terminals change its vesicular make up with respect to the transporter, although we cannot exclude regional differences. Regional differences may also be the reason that VGLUT2 varied only slightly but not significantly compared with the other vesicular proteins. VGLUT1 is the major transporter of the cortex and hippocampus, brain regions involved in activity-dependent behavior, learning, and memory, whereas VGLUT2 mainly occurs in the thalamus more related to unconscious functions. Probably *Per2* modulates VGLUT1-dependent presynaptic terminals involved in active tasks of the brain by switching between rest and activity corresponding to low or high amounts of VGLUT1 on SV and the respective time of the day.

The oscillation of VGLUT1 may be triggered by a diffusible oscillatory signal from the suprachiasmatic nucleus. As diffusible signals retinoic acid, transforming growth factor α , and prokineticin 2 are released from the suprachiasmatic nucleus (19, 20). Prokineticin 2 expression depends on light and is altered following shifts of the LD cycle (21). So changes in prokineticin 2 expression or its receptor may mediate the changes in the vesicular VGLUT1 content in SV from mice kept under DD compared with LD condition. In addition circadian variations of metabolic coupling between neuronal activity and energy substrate have been reported (22), which may directly change the efficiency of the presynaptic terminal by modulating the VGLUT1 content in the SV pools.

At the level of the glutamatergic neurons, *in situ* hybridization studies revealed no change in the expression of the VGLUT1 mRNA indicating that transcription of the transporter is not altered. The comparison of vesicular proteins in homogenate and synaptosomal preparations of wild type and *Per2^{Brdm1}* mutant mice also revealed similar amounts, implying that the total amount of synaptic proteins did not change in the starting fractions of SV. Collectively, these data indicate that synthesis and degradation of VGLUT1 were not altered.

Generally, the strength of synaptic activity defined by the size of the postsynaptic response depends on both pre- and postsynaptic factors contributing to short term and long term forms of synaptic plasticity. On the presynaptic site, release probability and transmitter content visualized by variations in either frequency or quantal size, respectively, are important factors. Vesicular content is directly linked to the amount of vesicular transmitter transporters (5) as also shown for VGLUT1 (7). The changes observed in the VGLUT1 content in the SV fraction therefore reflect changes in the synaptic output of VGLUT1-type neurons. The oscillation of VGLUT1 during LD cycles differs from the one obtained in mice kept under complete darkness and is disturbed in *Per2^{Brdm1}* mice. The changes in the amount of VGLUT1 protein are also

reflected by a functionally reduced glutamate uptake in the respective vesicle fractions. The reduction in the uptake was less expressed as one would expect from the blots; however, both VGLUT1 and VGLUT2 activity contribute to the amount of glutamate taken up by these fractions. It is possible that VGLUT1 associates or redistributes to vesicles less competent for glutamate uptake, *i.e.* compartments involved in the biogenesis of SV, although we did not observe changes in the vacuolar proton pump necessary for VGLUT function. In addition there may be also circadian and *Per2*-mediated changes in the chloride regulation of the VGLUTs affecting their transport efficiency (17).

It may be advantageous for the presynaptic terminal not to rely on an up- and down-regulation of the synthesis of VGLUT1 to modulate synaptic efficiency but rather provide vesicles of different VGLUT1 content. This implies that VGLUT1 as an integral membrane protein is sorted out of the SV pool and may be stored in another membrane pool at the respective time of the day. In this respect SV with the same amount of VGLUT1 may shift thereby increasing or decreasing storage capacity of the SV pool. Alternatively the amount of VGLUT1 per vesicle will change providing SV with either low or high concentrations of VGLUT1. Our data indicate that these membrane switches are influenced by circadian rhythms with the *Per2* gene playing a critical role. Probably the regulation of membrane fusion is not involved being the same for filled or empty vesicles (5) as also found for VGLUT1 deletion mutants (7).

In a given presynaptic terminal a small number of active vesicles are already docked to the plasma membrane and capable of rapid recycling undergoing repeated very fast rounds of exocytosis. The majority of vesicles are however present in the resting pool of vesicles which may participate less in secretion under regular conditions (Refs. 23, 24, and 25) (and references therein)). Synaptic vesicle-associated proteins like Rab3 (26, 27) and synapsin (Ref. 28 and references therein) appear to be involved in the organization of presynaptic pools. Studies have described a role of Rab3 and synapsin in regulation of circadian rhythms (29). However, neither Rab3 nor synapsin association to SV did change under our experimental condition. In this respect it is noteworthy that a genetic ablation of VGLUT1 resulted in a 50% decrease of synapsin and Rab3 in vesicular fractions suggesting that the synthesis of these proteins is linked to VGLUT1 expression (6), whereas during circadian rhythm the changes do not occur at the level of protein synthesis.

Recent data provide evidence that the active vesicles comprising the readily releasable pool are not necessarily only those near the plasma membrane but instead are dispersed randomly in vesicle clusters. A position at the surface of these vesicle clusters appears to favor recruitment for release (30). This indicates that there are no differences in the integral vesicular proteins in between the SV fractions and that the circadian-dependent changes in VGLUT1 may be attributed to all the different vesicular pools. As a consequence the reduced amounts of VGLUT1 at ZT6 and ZT12 will also lead to a reduced transmitter release at the majority of VGLUT1 terminals.

Because with the exception of VGLUT1 none of the other vesicular membrane proteins did change, the light- and *Per2*-dependent changes in the VGLUT1 amount of SV appears to be mediated by a so far unknown specific sorting mechanism. In this respect VGLUT1 can be sorted out either to endosomes or the plasma membrane, two membrane compartments targeted by SV during their life cycle. However, the recycling of SV during their exocytosis/endocytosis rounds is a very rapid process, and SV fractions may contain endosomal membranes and immature vesicles as well so that endosomal sorting out of VGLUT1 is less likely. However, SV fractions are generally devoid of plasma membrane contaminations. VGLUT1 besides being a vesicular glutamate

transporter may also work as a Na/PO₄ cotransporter when getting access to the plasma membrane (31). So the observed oscillation may reflect a switch for the VGLUT1 protein from a vesicular to a plasma membrane protein resulting in a specific decrease of VGLUT1 content of SV without changing the other vesicular properties. Whether VGLUT1 is sorted to the plasma membrane and how this sorting is controlled at the protein level remain to be determined. However, such circadian-dependent membrane traffic may allow the presynaptic terminal to replenish during physiological rest periods. It may also be speculated that the presynaptic terminal uses a similar mechanism under other paradigms, *i.e.* to avoid prolonged or repeated periods of enhanced stimulation.

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