Astrocytes contribute to gamma oscillations and recognition memory

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Glia cells are an integral part of functional communication in the brain. Here we show that astrocytes contribute to the fast dynamics of neural circuits that underlie normal cognitive behaviors. In particular, we found that the selective expression of tetanus neurotoxin (TeNT) in astrocytes significantly reduced the duration of carbachol-induced gamma oscillations in hippocampal slices. These data prompted us to develop a novel transgenic mouse model, specifically with inducible tetanus toxin expression in astrocytes. In this in vivo model, we found evidence of a marked decrease in electroencephalographic (EEG) power in the gamma frequency range in awake-behaving mice, whereas neuronal synaptic activity remained intact. The reduction in cortical gamma oscillations was accompanied by impaired behavioral performance in the novel object recognition test, whereas other forms of memory, including working memory and fear conditioning, remained unchanged. These results support a key role for gamma oscillations in recognition memory. Both EEG alterations and behavioral deficits in novel object recognition were reversed by suppression of tetanus toxin expression. These data reveal an unexpected role for astrocytes as essential contributors to information processing and cognitive behavior.

Significance

Astrocytes are well placed to modulate neural activity. However, the functions typically attributed to astrocytes are associated with a temporal dimension significantly slower than the timescale of synaptic transmission of neurons. Consequently, it has been assumed that astrocytes do not play a major role in modulating fast neural network dynamics known to underlie cognitive behavior. By creating a transgenic mouse in which vesicular release from astrocytes can be reversibly blocked, we found that astrocytes are necessary for novel object recognition behavior and to maintain functional gamma oscillations both in vitro and in awake-behaving animals. Our findings reveal an unexpected role for astrocytes in neural information processing and cognition.


The authors declare no conflict of interest.

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We conducted our experiments in transfected brain slices and in an inducible mouse model with temporally controlled and cell-specific expression of tetanus neurotoxin (TeNT), which we developed for this study. Our results uncover an unsuspected role for glial cells in neuronal network gamma oscillations and demonstrate their contribution to shaping higher brain functions and behavioral patterns.

**Results**

**Transient Elevations in Intracellular Calcium Concentration in Astrocytes Precede the Onset of Oscillatory Activity.** Synchronized fast neuronal oscillations in the beta (β, 12–20 Hz) and gamma (γ, 25–60 Hz) ranges have been described in association with perceptual and cognitive activities (15, 16). Neuronal oscillations can be induced by a variety of protocols (17, 18) and have been extensively studied in vitro, especially in hippocampal slices. In this work, we used cholinergic agonist carbachol (CCH) to induce oscillations in hippocampal slices. It has previously been shown that carbachol induces gamma oscillations in hippocampal slices by recruiting coordinated excitatory and inhibitory transmission in neural circuits (17). Using simultaneous imaging of intracellular calcium dynamics in astrocytes, loaded with the calcium sensor Fura-2 AM, and recording of field potentials in brain slices, we observed a temporal correlation between intracellular calcium elevations in astrocytes and field oscillatory activity (n = 6) (Fig. 1A–C). The onset of the calcium response in astrocytes preceded the start of the oscillations by 5.3 ± 2.4 s (mean ± SEM). The traces in Fig. 1B were taken from Fura-2–loaded cells, which were confirmed to be astrocytes at the end of the experiment by postfixation immune-labeling with anti-GFAP antibodies (Fig. 1D). This correlation prompted further investigations aimed at establishing whether astrocytic calcium responses were necessary for neural oscillations, or simply an epiphenomenon.

**Tetanus Toxin Expression in Cultured Astrocytes Disrupted Cell-to-Cell Signaling.** Because intracellular calcium elevations have been reported to be sufficient to induce glutamate release from astrocytes (19–21), we hypothesized that astrocytes might contribute to network oscillations by affecting the overall actions of glutamate and by modulating the excitability of the neuronal network. To investigate this possibility, we took advantage of the sensitivity of astrocytic calcium-dependent vesicular release to clostridial toxins (TeNT) (20, 22) and generated a genetic system capable of targeting TeNT expression to astrocytes. A synthetic gene coding for the TeNT catalytic domain (TeNT light chain, TeNT-LC) (23) was fused with the gene for green fluorescent protein (TeNTΔ1-GFP; Methods) to track toxin expression. Both wild-type and GFP-fused toxins can efficiently cleave synaptobrevin2, thereby preventing vesicle release (24).

We verified the ability of TeNTΔ1-GFP to inhibit glutamate release from astrocytes by using human embryonic kidney (HEK) cells made responsive to glutamate by the expression of the NMDA receptor subunits NR1 and NR2B (HEK-NMDA) (25, 26) (Fig. 2A–E). These HEK-NMDA cells were plated on top of primary astrocyte cultures that had been transfected with either GFP (Fig. 2 A–C) or TeNTΔ1-GFP cDNAs (Fig. 2 D and E). Thus, in this preparation, HEK-NMDA cells were able to detect glutamate released from astrocytes. Mechanical stimulation of untransfected astrocytes, or astrocytes transfected with GFP cDNA, induced a calcium response in all HEK-NMDA cells located within a 150-μm radius from the stimulation site (ΔF/F = 0.3 ± 0.08, mean ± SEM; n = 21 cells from three cultures) (Fig. 2B). Note that CCH could not be used in place of mechanical stimulation in these experiments because it also directly induced elevated calcium levels in HEK cells. Calcium responses in HEK-NMDA cells following mechanical stimulation of astrocytes were completely abolished by the NMDA receptor-antagonist APV (50 μM) (n = 15 cells from three cultures) (Fig. 2C).

In contrast, when astrocytes were transfected with TeNTΔ1-GFP, mechanical stimulation did not result in a response in HEK-NMDA cells (n = 16 cells from three cultures) (Fig. 2E), presumably due to the impairment of vesicular release in astrocytes expressing the toxin. These results were consistent with previous reports (20, 21, 27, 28) and validated the sensitivity of astrocytic vesicular release to tetanus toxin block.

**In Brain Slices, Astrocystic Expression of Tetanus Toxin Blocked Glutamate Release Without Interrupting Neuronal Synaptic Activity.** With the ultimate goal of expressing the TeNTΔ1-GFP construct in astrocytes of brain slices, we engineered a lentiviral vector with the human astrocyte-specific promoter hGFAP (29) and a GFP-only control (Fig. 2F). We first infected primary cultures of astrocytes with the control virus (lenti-hGFAP-GFP) and then cultured HEK-NMDA cells on top as previously described. We found that the infection and subsequent GFP expression was successful, yet had no effect on astrocytic glutamate release (fluorescence changes in HEK cells within 150 μm from stimulation were the following: ΔF/F = 0.4 ± 0.09, mean ± SEM; n = 8 cells from three cultures) (Fig. 2 G and H). In contrast, infection with lenti-hGFAP-TeNTΔ1-GFP effectively blocked glutamate release from astrocytes by the same measure (n = 12 cells from three cultures) (Fig. 2 J).

**Fig. 1.** Intracellular calcium responses precede carbachol-induced oscillations. (A) Immunofluorescence-based profiling of the three cells imaged in B. (Top) Fura-2–loaded cells (white arrows) visualized using a 380-nm excitation wavelength. (Middle) The slice was fixed and immunostained using a rabbit anti-GFAP antibody. (Bottom) The merge of the Left and Center panels shows that the Fura-2–loaded cells were GFAP-positive astrocytes. To minimize neuronal loading, short Fura-2–loading time was used, and consequently only a few cells were loaded with the calcium dye. (B) Example traces of intracellular calcium dynamics in three astrocytes from stratum radiatum of the CA3 area following carbachol treatment (CCH). (C) Extracellular field potential from the pyramidal cell layer of the CA3 area recorded simultaneously with the calcium imaging shown in B. Note that the onset of the calcium response preceded the start of the oscillations. (D) Gamma oscillations from recording in C shown on expanded time scale.
The Tetanus toxin expression in astrocytes eliminated the release of glutamate but did not significantly modify synaptic activity. The glutamate detectors (HEK-NMDA), the HEK cells with NR1 and NR2B, were seeded on top of primary astrocyte culture (A), and the HEK-NMDA cell responses to mechanical stimulation of astrocytes were measured using a fluorescent calcium indicator. (A) Merged bright-field and dsRed channel images. Transfected HEK cells are visualized by virtue of dsRed protein expression (red arrows). White arrow is the stimulation site. Numbered circles are astrocytes where the intracellular calcium dynamics were monitored. (B) Intracellular calcium concentration in astrocytes (blue and purple traces) and HEK-NMDA cells (red traces) following mechanical stimulation. (C) The experiment described in A and B was repeated in the presence of 50 μM AP-5. (D and E) Intracellular calcium concentration following transfection of astrocytes with TeNTα1-GFP cDNA. TeNTα1-GFP-transfected cells were visible with green florescent color (D); blue and purple traces are the astrocytes response to mechanical stimulation, whereas the HEK-NMDA cells did not detect any extracellular glutamate (E, red traces). (F) Schematic representation of the lentiviral vector constructs used in this study. (G and H) Infection of astrocytes with lenti-GFP. Red arrow indicates HEK-NMDA cell. Black arrow indicates stimulation sites. Intracellular calcium concentration (H) in astrocytes (black trace) and HEK-NMDA cells (red trace) following mechanical stimulation of the astrocyte. (I and J) Infection of astrocytes with lenti-TeNTα1-GFP with HEK-NMDA. Note that HEK-NMDA did not detect glutamate from the astrocytes infected with TeNTα1-GFP. (K–M) Targeted expression of TeNT to astrocytes in slice cultures had no significant effect on synaptic activity. Traces show mEPSCs recorded from pyramidal neurons in the CA3 region infected with astrocyte-targeted lentivirus containing GFP (K, Upper) and TeNTα1-GFP (K, Lower). (L) Mean amplitude of mEPSCs was 17.03 ± 1.64 pA (n = 3) and 18.51 ± 1.67 pA (n = 3) for GFP and TeNTα1-GFP, respectively. (M) Mean frequency of mEPSCs was 0.23 ± 0.046 Hz (n = 3) and 0.22 ± 0.002 Hz (n = 3) for GFP and TeNTα1-GFP, respectively.

We next used the viral vectors in primary hippocampal slices and validated the targeting expression to astrocytes. Using immunohistochemistry, we found that GFP expression colocalized with a marker for astrocytes (GFAP), but not with one for neurons (NeuN) (Fig. S1A). Additionally, field-excitatory postsynaptic potentials recorded from infected cultured slices, expressing GFP alone or TeNTα1-GFP, were indistinguishable from each other (Fig. S1 B and C), suggesting that this measure of neuronal function was intact. Finally, we studied synaptic activity by recording miniature excitatory postsynaptic currents (mEPSCs) and found that pyramidal neurons located in the region infected with the lentiviral vectors showed normal mEPSCs (Fig. 2 K–M), again indicating normal function in these cells. Taken together, these results suggest that, when vesicle fusion in astrocytes was blocked by TeNTα1-GFP in our model, neuronal synaptic activity remained largely unaltered and that there was no leaky expression of tetanus toxin in neuronal cells.

Blockade of Astrocytic Vesicular Release Shortened the Duration of Carbachol-Induced Gamma Oscillations in Vitro. Next we asked whether TeNTα1-GFP expression in astrocytes affected the neurophysiology of hippocampal slices. We first looked at network oscillations in slices that had been maintained in culture for at least 5 d and were either uninfected or infected with our control lenti-GFP (Fig. 3A). Bath application of carbachol (30–50 μM) induced fast gamma and beta oscillations (Fig. 3 B and E), similar to what has previously been reported in acute hippocampal slices (17, 18). However, when slices were infected with lenti-TeNTα1-GFP, carbachol induced only short-lasting gamma oscillations (Fig. 3C). The duration of gamma oscillations in uninfected and lenti-GFP–infected slices was 1.9 ± 0.4 s (mean ± SEM; n = 9) and 1.7 ± 0.2 s (mean ± SEM; n = 12), respectively. In contrast, in lenti-TeNTα1-GFP–infected slices, the duration of carbachol-induced gamma oscillations was reduced to 0.3 ± 0.07 s (mean ± SEM; n = 16) (Fig. 3F, F1). Other parameters of gamma oscillations (number of events, amplitude, and peak oscillation frequency) were not sensitive to toxin expression (Fig. 3F, F2–F4). Additionally, no alteration was observed in beta oscillations (Fig. 3F, F5–F8), suggesting that the effect of TeNTα1-GFP expression was specific to oscillations in the gamma frequency range. Next, using intracellular recordings, we measured the effect of astrocyte-specific TeNT expression on the depolarization of pyramidal cells induced by carbachol. In cultured slices infected with lenti-GFP, carbachol application resulted in large depolarizations of pyramidal neurons (18 ± 5 mV, mean ± SEM; n = 6), whereas expression of lenti-TeNTα1-GFP resulted in smaller carbachol-dependent depolarizations (6 ± 1 mV, mean ± SEM; n = 7; P = 0.02, Student’s t test) (Fig. S2).

To eliminate the possibility that interslice variation caused some of the differences observed between control and lenti-TeNTα1-GFP–infected slices, we infected some slices (n = 4) with a limited amount of lenti-TeNTα2-GFP and restricted the infection to one small region. This allowed an internally controlled comparison of oscillatory behaviors inside and outside the infected area within the same slice. The duration of gamma oscillations recorded within the infected area was, in all cases, significantly shorter than the duration of gamma oscillations recorded outside the infected region (Fig. 3G).

Glutamate Receptor Agonist AMPA Reversed the Disruption of Gamma Oscillations. To test whether the shortening of gamma oscillations in TeNTα1-GFP–infected slices involved the impaired release of glutamate from astrocytes, we determined whether the exogenous application of the glutamate receptor agonist AMPA could reverse the effect of the toxin. Low concentrations of AMPA (10–30 μM) by themselves did not induce oscillatory activity at any frequency when applied to the slices. However, when carbachol (30 μM) and AMPA (20 μM) were coapplied to the lenti-TeNTα1-GFP–infected slices, the duration of the gamma oscillations was restored to control values (2.1 ± 0.5, mean ± SEM; n = 8) (Fig. 3D and F, F1). These results showed that the integrity of the neural circuits responsible for gamma oscillations was intact in slices with astrocytic TeNTα1-GFP expression and that the shortening of the oscillations was not due to secondary effects of viral infection or to toxin expression. Furthermore, these findings suggest that glutamate could be a glia-released factor responsible for extending the otherwise shortened duration of gamma oscillations.

Development of an Inducible Transgenic Mouse Model Allowing Temporal and Cell-Specific Control of TeNT Expression in Astrocytes. Next, to find out whether the findings of astrocyte-mediated modulation of gamma oscillations in vitro had any physiological
or behavioral relevance in vivo, we generated a transgenic mouse line expressing TeNT in astrocytes. Our attempt to use the same lenti-GFAP-TeNT\(^{31}\)-GFP vector (Fig. 2F) for creating a transgenic mouse (30) failed to generate a mouse line with stable TeNT expression. We reasoned that the constitutive expression of the toxin in astrocytes might have been lethal to the animals.

We then developed a system in which astrocytic expression of TeNT\(^{31}\)-GFP could be controlled temporally. The transgene that we used contained two control elements: a tetr-responsive element (TRE) promoter (31) that is active only when a tet-transactivator (tTA) is bound and a loxP-flanked transcriptional/translational STOP sequence (32), which prevented the expression of tetanus toxin unless it was excised by Cre recombinase. Hence, we created the transgene TRE-loxP-STOP-loxP-TeNT\(^{31}\)-GFP (for short, TRE-STOP-TeNT-GFP) and subsequently crossed it with the previously reported transgenic mouse lines GFAP-tTA (33) and GFAP-CreER\(^{2}\) (34). CreER\(^{2}\) is a fusion protein of the estrogen receptor to Cre recombinase that re-

![Image](https://www.pnas.org/cgi/doi/10.1073/pnas.1410893111)
frequency ranges and in all behavioral states (Fig. 6 C–E, Bottom Left); such an effect could be reversed by doxycycline administration (3×TG-T+D) (Fig. 6 C–E, Upper Right and Lower Center).

Statistical analysis (ANOVA) on measured EEG power spectra revealed that the only significant change associated with induction of TeNT expression was in the low-gamma range (20–40 Hz, maximum ~30 Hz, Fig. 6C, Upper) when the animals were awake. During non-REM and REM sleep, however, no significant alteration of the oscillations was observed (Fig. 6 D and E, Upper). Comparison of EEG power spectra before and after doxycycline treatment showed a similar state-dependent spectral modulation in 3×TG mice, but not in control animals (Fig. 6 C–E, Right).

**Astrocyte-Specific TeNT Expression Led to Behavioral Changes in Novel Object Recognition.** We then conducted a battery of cognitive tests to investigate if the blockade of astrocytic vesicular release led to behavioral abnormalities. The following paradigms were used (in order of testing sequence): Y-maze, novel object recognition (NOR), and fear conditioning (FC, cued and contextual).

We observed a significant memory deficit in the NOR test (Fig. 7C). Wild-type mice naturally spend more time exploring a new object than a familiar one. If a mouse has defective recognition memory, it would be expected to spend equal or more time with the familiar object. The discrimination index \(\text{DI} = (T_{\text{NOVEL}}-T_{\text{FAMILIAR}})/(T_{\text{NOVEL}}+T_{\text{FAMILIAR}})\) was used to quantify the relative exploration time for the novel versus the familiar object (43). The DI can vary between −1 and +1, where positive scores indicate more time spent with the novel object and a zero or negative score indicates equal or less time spent with the novel object, which would be considered a defective recognition memory (44). Whereas the control (2×TG-T) mice showed significant preference for the novel object, the 3×TG mouse treated with tamoxifen (3×TG-T, i.e., TeNT expressed in astrocytes) showed a DI value of almost zero, indicating defective recognition memory. However, this defective recognition memory was restored by suppression of TeNT expression by doxycycline feeding (3×TG-T+D), suggesting that gamma oscillations were required for the normal functioning of this type of recognition memory.

In contrast, we found no impairments in Y-maze or FC tests. When mice are tested for navigational working memory using the Y-maze test, they typically prefer to investigate a new arm of the maze rather than return to one that was recently visited. Data were analyzed to determine the number of alternating arm entries without repetition (unique triplets). As shown in Fig. 7A, TeNT-expressing mice did not show changes compared with the control groups. Similarly, no significant differences were observed in associative fear memory in a conditioned fear task.

In this test, Pavlovian associative learning was evaluated as freezing responses to the conditioned context environment or a previously neutral stimulus (a tone cue) associated with an aversive stimulus (electric foot shock). Freezing levels were very low across all groups in the context test, making group comparisons difficult. Although there was an overall increase in freezing in this trial relative to the habituation trial, no statistically significant group differences were observed. All control and experimental mouse groups exhibited the expected increase in freezing in the conditioned stimulus (CS) + test upon exposure to the shock-associated cues (Fig. 7B).

**Discussion**

In this study, we used molecular genetic tools to specifically perturb the activity of astrocytes in vitro and in vivo. We induced the expression of TeNT in these cells, taking advantage of the fact that this toxin can efficiently prevent vesicle fusion with the plasma membrane. Using transfected brain slices, we first discovered that vesicular release in astrocytes is required for the maintenance, but not the triggering, of gamma oscillations. Then, using a novel inducible mutant mouse model, we found that TeNT expression in astrocytes reduced EEG power density in the gamma frequency range in vivo. The consistency of findings between different preparations strongly supports the broad and unexpected contribution of astrocytes to gamma oscillatory rhythms, which were previously thought to depend exclusively on neuronal activity. Finally, our results suggest that intact vesicular release in astrocytes and the ensuing disruption of gamma oscillatory activity can profoundly impact cognition, as reflected by the deficit in recognition memory.

Several models of carbachol-induced gamma oscillations in the hippocampus have been previously described (45–47), which postulated a tonic depolarization of excitatory and inhibitory neurons. However, all previously proposed models assumed that such tonic depolarization would have an exclusively neuronal origin and none of them considered the possible involvement of astrocytes. Our results, on the contrary, indicate that intact vesicle trafficking in glial cells is in fact an important part of neuronal network dynamics. Although carbachol-induced depolarization might be sufficient to trigger gamma oscillations, a larger depolarization was required to maintain the oscillatory activity, which could be obtained by recruitment of astrocytes and the consequent release of transmitter from these cells.

Although these data support glutamate as a possible gliotransmitter, our genetic model was not specific in this regard. Sustained TeNT expression in astrocytes can be expected to inhibit the trafficking of all vesicles, potentially blocking the delivery of a variety of channels and transporters to the plasma membrane (48). This could impact the K⁺ buffering and glutamate uptake functions of astrocytes, which are critical for normal neuronal network dynamics. Similarly, the ability of TeNT expression in astrocytes to affect carbachol-induced oscillations could result from the reduced surface expression of muscarinic
\textbf{Fig. 5.} Synaptic responses were preserved in astrocytic TeNT-expressing transgenic mice. (A) Input/output relationship between the fiber volley amplitude and fEPSP slope was determined over a range of stimulus intensities. Each point represents the mean of all slices for each stimulus intensity. Error bar illustrates SEM for both axes (\(n_{2xTG}\) = 15 slices from seven mice, \(n_{3xTG}\) = 17 slices from eight mice). (B) Paired-pulse facilitation induced by two consecutive stimuli (S\(_1\) and S\(_2\)) delivered at different time intervals. Facilitation is represented as the S\(_2\)/S\(_1\) ratio (one slice per mouse, \(n = 6\) mice each). (C) Sustained fatigue induced by 12 consecutive stimuli at 25-ms inter-stimulus intervals (one slice per mouse, \(n = 6\) mice each). (D) Long-term potentiation induced by high frequency (100 Hz for 1 s repeated three times at 5-min intervals, as indicated by the arrows; one slice per mouse, \(n = 5\) mice each). (E) Long-term depression induced by low-frequency stimulation (1 Hz for 15 min, \(n_{2xTG}\) = 4 slices from three mice, \(n_{3xTG}\) = 3 slices from three mice). (F) mEPSCs obtained in the presence of TTX (0.5–1 \(\mu\)M) and picrotoxin (100 \(\mu\)M) with demonstrative recordings (Upper). (Lower) Mean amplitude/frequency \(\pm\) SEM. \(n_{2xTG}\) = 14 cells from five mice, \(n_{3xTG}\) = 9 cells from three mice. Demonstrative FEPSP traces are illustrated as insets in B–E. (Scale bar, 10 ms and 0.5 mV.)

Acetylcholine receptors, consequent to impaired vesicular trafficking. Although these alternative explanations remain possible, exogenous application of the glutamate receptor agonist AMPA was capable of rescuing the duration of oscillations that were reduced with defective vesicle fusion in our model. Coupled with recent evidence that perisynaptic astrocytes express the vesicular glutamate transporters VGLUT1 and VGLUT3 (49, 50) and that glutamate accumulates in synaptic-like microvesicles in the perisynaptic processes of astrocytes at a concentration comparable to that in synaptic vesicles of excitatory nerve terminals (51), glutamate may be said to in some way mediate the impact of astrocytes on network dynamics.

The fact that the expression of tetanus toxin did not cause evident changes in basal synaptic transmission, as measured in cultured and in acute slices, strongly suggested that the TeNT gene, delivered by lentiviral vectors in slice cultures or integrated into the genome of transgenic mice, was exclusively expressed in astrocytes and not in neurons. Because of their proximity to neuronal synapses, as well as their ability to detect neuronal firing and respond with cytosolic Ca\(^{2+}\) elevations, it has been proposed that astrocytes might play an active role in synaptic plasticity (52). Following the induction of TeNT expression, we did not observe appreciable effects on short-term or long-term synaptic plasticity. This is in line with results obtained in a different mouse model in which astrocytic calcium signaling was impaired (14). However, the lack of effect on basal synaptic transmission was in contrast with results obtained in another study using dominant negative SNARE (dnSNARE) mice (33). In the dnSNARE transgenic mouse line, the dnSNARE, amino acids 1–96 of synaptobrevin II, was subcloned under the tetO promoter, which was silenced by continuous doxycycline feeding from parental mating to 6–8 wk of age. The difference observed between the dnSNARE and our TeNT expression mouse lines might be due to (i) different genetic tools used to control the expression of the transgene (continuous doxycycline treatment versus tamoxifen activation), (ii) the use of genetically controlled enzymatic degradation of vesicular-release machinery versus controlled expression of a dominant negative peptide, and (iii) different levels of expression of the transgene and overall efficiency of vesicular release inhibition in the two systems. It should also be noted that we aimed primarily at controlling possible effects of leaky expression of tetanus toxin in neurons. As such, although our data suggest that synaptic mechanisms were largely intact in our model, protocol-specific contributions of vesicular release to synaptic plasticity cannot be ruled out.

Our in vivo recordings revealed a significant decrease of EEG power density in the low-gamma-frequency range (25–40 Hz) of astrocytic TeNT-expressing mice. The gamma power density reduction was reverted by suppressing the TeNT transgene expression by feeding the animals with doxycycline. Together, these findings demonstrate that TeNT-sensitive vesicular release from astrocytes is required for sustaining gamma oscillations. Interestingly, our results demonstrate that astrocytic TeNT-induced disruption of low gamma oscillations (compared with other frequency ranges) was significant only when the animals were awake, but not during sleep. Such a behavioral state-dependent effect suggests that astrocyte-sustained gamma oscillations could be especially critical for active cognitive functions, such as performance in the novel object recognition test, which evaluates the integrity of recognition memory.
We have used several behavioral tests to investigate the relationship between gliotransmission-dependent gamma oscillations and cognitive functions and behavior. Interestingly, after sequentially testing the same mice in the Y-maze, novel object recognition, and fear conditioning tests, we found that astrocytic TeNT expression resulted in significant deficits in the NOR test, with no differences observed in the Y-maze and fear-conditioning tests. These results indicate that the animals did not have global deficits in attention, sensory functions, or exploratory drive. The deficit in NOR performance, on the other hand, paralleled the alterations in EEG, and like the EEG deficits, it was fully rescued after suppressing the expression of TeNT. Although both the Y-maze task and the NOR test rely on the rodent’s innate exploratory behavior in the absence of externally applied positive or negative reinforcement, defects were selectively observed in the case of the NOR test. This is particularly relevant because the Y-maze task evaluates a simpler form of learning involving a sympathetic reflex reaction to novel stimuli, whereas NOR involves a higher memory load engaging long-term storage, retrieval, and restorage of memory processing. During the test phase of the NOR test, a novel object needs to be detected and encoded, whereas the memory trace of a familiar object needs to be updated and reconsolidated after long delays. In contrast, fear conditioning might constitute a strong and highly specific form of learning involving a sympathetic reflex reaction with suppression of voluntary movements (freezing), in which subtle changes in memory content might not be detectable. Moreover, there is strong evidence that suggests fear-conditioned learning encodes a long-term memory process involving the amygdala and the hippocampus, whereas the NOR paradigm engages different structures: the hippocampus and adjacent...
spontaneous alternations (unique triplets) (3). These groups were different [Fisher discriminate between the familiar and the novel object, and therefore (Y-maze)] E3350 oscillations are not simply an epiphenomenon of neuronal of gamma oscillations provide evidence that fast network behavior deficits that we have observed following the disruption regulated by astrocytes. Furthermore, the cognitive and be-

In conclusion, we have shown that astrocytic vesicular release for the first time, that fast neural circuit oscillations are tightly for the first time, that fast neural circuit oscillations are tightly

Acute Slices and Electrophysiology. Hippocampal slices (350-450 μm thick) were prepared from (i) 8- to 12-d-old C57BL/6J mice (and then lentiviral-transfected) or (ii) 14- to 17-d-old (TeNT transgenic) mice in sucrose-based ice-cold solutions bubbled with 95% (vol/vol) O2 (95% (vol/vol) CO2), respectively, containing the following (in mM): (i) sucrose, 200; KCl, 1.9; NaH2PO4, 1.2; dextrose, 10; NaHCO3, 33; CaCl2, 0.7; MgCl2, 4; ascorbic acid, 0.4; or (ii) 110 sucrose, 2.5 KCl, 0.5 CaCl2, 7 MgCl2, 25 NaHCO3, 1.25 NaH2PO4, and 10 glucose. The slices were then transferred to artificial cerebrospinal fluid (aCSF) composed of (in mM): (i) NaCl, 124; KCl, 5; CaCl2, 2; MgCl2, 2; NaH2PO4, 1.2; dextrose, 10; NaHCO3, 33; or (ii) 130 NaCl, 2.5 KCl, 1.25 NaH2PO4, 24 NaHCO3, 10 glucose, 2 CaCl2, and 1.3 MgCl2, equilibrated with 95% (vol/vol) O2/5% (vol/vol) CO2. The tissue was then transiently incubated for 30 min at 32 °C (skipped for transfected slice experiments) and afterward kept at room temperature (24 °C) for ≥1 h before use. Extracellular recordings were obtained using glass microelectrodes filled with aCSF (resistance 2–6 MΩ) in submerged chambers maintained at 32 ± 1 °C (transfected slices) or at room temperature (24 °C, TeNT line). Data were acquired using an Axopatch 200B amplifier or a Multiclamp 700B and pCLAMP software (Axon Instruments) or the WinLTP program (S6).

In experiments involving TeNT mouse line, field-excitatory postsynaptic potentials (fEPSPs) were evoked by stimulation of the Schaffer collateral-commisural fibers with rectangular 0.2-ms pulses delivered through a concentric bipolar electrode and recorded in the stratum radiatum of the CA1 hippocampal region. Input–output curves were constructed by increasing the stimulation intensity. fEPSPs were evoked each 15 s (0.067 Hz), except for paired-pulse facilitation experiments in which pairs of stimuli (25- to 300-ms intervals) were delivered at 0.33 Hz to avoid interferences with slower short-term potentiation (augmentation) or depression processes (57). For long-term potentiation (LTP) and long-term depression (LTD) experiments, fEPSPs were reduced to ~40–60% or 60–80% (10-30 Hz stimulation intensity), respectively, and stable baselines of at least 20 min were collected. LTD was induced by low-frequency stimulation (900 stimuli at 1 Hz); LTP was induced by high-frequency stimulation (100 Hz for 1 s repeated three times at 5-min intervals), and the experiments were validated if the fiber volley did not vary significantly. Synaptic fatigue was accessed by 12 consecutive stimuli delivered at 40 Hz.

mEPSCs were recorded at 5 kHz and filtered at 2 kHz from CA1 pyramidal neurons in voltage-clamp mode [voltage holding potential (VH) = −70 mV] in the presence of TTX (0.5–1 μM) and picrotoxin (100 μM). Events larger than 5 pA were accepted using a low-pass filter (10 kHz) and a high-pass filter (40 kHz) and are plotted as 

Calcium Imaging. Hippocampal slices were incubated 30–50 min at room temperature in aCSF saturated with 95% (vol/vol) O2/5% (vol/vol) CO2 and containing 10 μM Fura2-AM (Molecular Probes). For imaging calcium dynamics in astrocytes at the interface between the astrocyte and the neuron, we used a calcium indicator with high temporal resolution. After the slices were loaded with the indicator, the slices were transferred to a perfusion chamber and the images were acquired using a confocal microscope (Olympus Fluoview). The images were analyzed using customwritten software (written by Dr. R. K. M. Siegel). The data were then normalized to the baseline fluorescence and the traces were analyzed using custom-written software (written by Dr. R. K. M. Siegel). The data were then normalized to the baseline fluorescence and the traces were analyzed using custom-written software (written by Dr. R. K. M. Siegel). The data were then normalized to the baseline fluorescence and the traces were analyzed using custom-written software (written by Dr. R. K. M. Siegel).

Slice Cultures. Horizontally hippocampal slices (400 μm thick) were cut from P9 mouse pups (C57BL/6J) in ice-cold calcium-free minimum essential medium
A third electrode was placed over cerebellum as ground. Two additional wire electrodes were placed in the cervical musculature for electromyogram (EMG) recordings of postural and insularized leads from these electrodes were then soldered to a miniconnector cemented to the skull with dental acrylic. The wound was sutured and treated with antibiotic ointment, and the animal was injected s.c. with fluorin (analgescic).

One week after surgery, animals were treated during 2 consecutive days with a single (i.p.) injection of tamoxifen (100 mL, 10 mg/mL). Recording sessions were performed 3 d after the second injection. Animals were first habituated in individual recording chambers for 48 h under standard temperature and light/dark cycle conditions. Then the animals were recorded continuously for 24 h starting after light onset. The EEG and EMG signals were amplified by a Grass Model 7D polygraph, filtered in a frequency range of 0.3–60 Hz, sampled at 256 Hz, and stored on a hard drive downsampled to 128 Hz for offline analysis, using software supplied by Kissei Comptec. Fifteen-second epochs of the polygraphic recordings were semiautomatically classified into three behavioral states: wakefulness, REM sleep, and non-REM sleep. Spectral analysis of EEG signals was performed by using fast Fourier transform on 4-s epochs and 1-Hz frequency bins. Epochs contaminated with artifacts as well as those occurring at transitions between behavioral states were discarded from analyses. For each behavioral state, i.e., wakefulness, REM sleep, and non-REM sleep, two-way ANOVA was applied to spectral power proportion for each frequency band. The first independent factor was genotype (2x versus 3x) and the second factor was treatment (tamoxifen versus tamoxifen+doxycycline). A significance level of $P = 0.05$ was used to identify frequency ranges in which marked differences in EEG power were observed between the two genotypes as well as between the two treatments.

**Y-Maze.** Spontaneous alternation, the tendency to alternate free choices in a Y-maze (three arms), was used to study working memory. A single 5-min test was performed in which each mouse was placed in the center of the Y. Total number of arm entries and the order of entries were determined from video-recorded sessions. Spontaneous alternations were defined as consecutive triplets of different arm choices (typical score for normal mice was 60–70%).

**NOR.** The NOR test assesses the ability to recognize a novel object in the environment. Adapted from Benice and colleagues (61, 62), mice were individually habituated to a 51- × 51- × 39-cm open field for 5 min. For the familiarization trials, two plastic toy objects were placed in the open field (one in each of two corners), and an individual animal was allowed to explore for 5 min. This was repeated another four times (1-min intertrial interval). Two weeks later, each mouse was tested in a NOR test in which a novel object replaced one of the familiar objects (no preference for any object was observed in pilot studies using C57BL/6J mice). All objects (fixed to 10–7 × 7–0.5-cm clear Plexiglas bases to prevent displacement) and the arena were thoroughly cleaned with 70% ethanol between trials to remove odors. “Exploration” was defined as approaching the object nose first (2.4 cm. The numbers of approaches toward each object were calculated for each trial. Habituation to the objects across the five familiarization trials (decreased contacts) is an initial measure of learning, and renewed interest (increased contacts) in the new object indicates evidence of object memory.

**Cued and Contextual Fear Conditioning.** Conditioning took place inside soundproofed boxes with a Mouse NIR Video Fear Conditioning System (Med Associates). The conditioning chambers (26 × 26 × 17 cm) are made of Plexiglas with speakers and lights mounted on two opposite walls and a shockable grid floor.

On day 1, mice were placed in the conditioning chamber for 5 min to habituate them to the apparatus. On day 2, the mice were exposed to the context (shock and CS) on 30 s, 3,000 Hz, 80 dB sound + white light) in association with foot shock (0.60 mA, 2-s, scrambled current). In each session, the mice received three shock exposures, each in the last 2 s of a 30-s tone/light exposure. Two weeks later, contextual conditioning (as determined by freezing behavior) was measured in a 5-min test in the chamber where the mice were trained (context test). On the following day, the mice were tested for cued conditioning (CS-test). The mice were placed in a novel context (white opaque plastic floor and walls creating a circular compartment, in contrast to a clear plastic square compartment and metal grid floor) for 3 min and then exposed to the CS for 3 min. Freezing behavior (absence of all voluntary movements except breathing) was measured in all sessions by real-time digital video recordings calibrated to distinguish between subtle movements (whisker twitch, tail flick) and freezing behavior. Freezing behavior in the context and cued tests (relative to the same environment or CS) and the shock.