Down syndrome (DS) in humans is caused by trisomy of chromosome 21 and is marked by prominent difficulties in learning and memory. Decades of research have demonstrated that the hippocampus is a key structure in learning and memory, and recent work with mouse models of DS has suggested differences in hippocampal activity that may be the substrate of these differences. One of the primary functional differences in DS is thought to be an excess of GABAergic innervation from medial septum to the hippocampus. In these experiments, we probe in detail the activity of region CA1 of the hippocampus using in vivo electrophysiology in male Ts65Dn mice compared with their male nontrisomic 2N littermates. We find the spatial properties of place cells in CA1 are normal in Ts65Dn animals. However, we find that the phasic relationship of both CA1 place cells and gamma rhythms to theta rhythm in the hippocampus is profoundly altered in these mice. Since the phasic organization of place cell activity and gamma oscillations on the theta wave are thought to play a critical role in hippocampal function, the changes we observe agree with recent findings that organization of the hippocampal network is potentially of more relevance to its function than the spatial properties of place cells.

Key words: Down syndrome; hippocampus; learning and memory; place cell; theta rhythm; Ts65Dn

Significance Statement

Recent evidence has disrupted the view that spatial deficits are associated with place cell abnormalities. In these experiments, we record hippocampal place cells and local field potential from the Ts65Dn mouse model of Down syndrome, and find phenomenologically normal place cells, but profound changes in the association of place cells and gamma rhythms with theta rhythm, suggesting that the overall network state is critically important for hippocampal function. These findings also agree with evidence suggesting that excess inhibitory control is the cause of hippocampal dysfunction in Down syndrome. The findings also confirm new avenues for pharmacological treatment of Down syndrome.

Introduction

The human chromosomal disorder Down syndrome (DS) produces a spectrum of interrelated morphologic changes, as well as deficits in executive function (Rowe et al., 2006; Lanfranchi et al., 2010). Individuals with DS exhibit deficits in learning and memory (Simon et al., 2009; Lott and Dierssen, 2010). The Ts65Dn mouse model provides replication of changes seen in human DS (Freeburn and Munn, 2021).

Ts65Dn mice show changes in output from medial septum (MS) to hippocampus (Kelley et al., 2014a,b). The MS is a major source of excitatory cholinerergic and glutamatergic, and inhibitory GABAergic input to the hippocampus (Costa et al., 1983; Gulyás et al., 1990; Hangya et al., 2009; Vandecasteele et al., 2014). The push-pull of excitation/inhibition is thought to be the key generator of local field potential (LFP) theta rhythm (Allen and Crawford, 1984; Brazhnik et al., 1985; Brazhnik and Vinogradova, 1986; Vinogradova, 1995; Vandecasteele et al., 2014). Theta is thought to segregate the flow of information within hippocampus into encoding or retrieval epochs (Hasselmo et al., 2002; Judge and Hasselmo, 2004; Hasselmo, 2005; Manns et al., 2007; Newman et al., 2013, 2017; Hasselmo and Stern, 2014; Schomburg et al., 2014). The phasic occurrence of place cell single-unit spiking activity on the
The maximal number of simultaneously recorded neurons was 11. The electrodes were placed at 1.6 mm lateral from the midline and anterior to bregma, and 1.8 mm posterior to bregma. Data were recorded online with a 1.5 ms sampling rate, and then processed offline using 1.5 ms windows. The electrodes were placed above the hippocampus and brainstem, and were recorded at 110 and 500 Hz for the hippocampus and brainstem, respectively.

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**Materials and Methods**

**Animals and surgery.** All experimental procedures were conducted in accordance with and were approved by the Institutional Animal Care and Use Committee at Stanford University School of Medicine. Male mice trisomic for the distal portion of chromosome 16 (Ts65Dn, n = 4) and their 2N (Control, n = 4) male littermates were used for single-unit recordings. These animals were between 3 and 9 months of age at time of surgery, and weighed between 25 and 35 g. Animals in each cohort were from the same litter, and so were age-matched. Animals were deeply anesthetized using isoflurane anesthesia (1.5%-2% in oxygen with flow rate 1200-1500 ml/min), and were given a subcutaneous injection of buprenorphine (0.03 mg). Animals were implanted with 16-channel tetrode carrying microdrives into the right hemisphere. The tetrodes themselves each consisted of four 17 mm platinum/iridium polyimide-coated electrodes twisted together. The electrodes were electropolated using Platinum Black (Neuralynx) until their impedances were similar and were in the region 200-250 kΩ. Coordinates for the electrodes were −1.8 mm posterior to bregma, and 1.6 mm lateral from the midline. Electrodes were implanted to an initial depth of 900-1000 mm, which typically placed them just dorsal to the hippocampal pyramidal cell layer. Animals were between 4 and 12 months old during recordings. Recordings were made during the animal’s daylight cycle.

**Statistics.** All statistics were two-sided. An Anderson–Darling test was used to determine whether the data were parametric. In the case of non-parametric data, either a signed-rank (paired) or rank-sum (unpaired) test was used. A summary of these tests of parametricity is presented in Table 1. If data were parametric, a two-tailed t test was used for single comparisons. Differences between noncircular distributions were determined using a two-sample Kruskal–Wallis test. Determination of circular clustering was done via Rao’s spacing test. A nonparametric multiasample test for equal circular medians was performed (Fisher, 1995). Where the observed incidence of a variable was compared against an expected value, a χ² test of proportions was used.

**Data acquisition.** Animals were screened for single units while they explored a 30 × 30 cm environment made of black polycarbonate. The walls of this enclosure were made unique through affixing a sheet of white paper to one wall, and alternating black and white diagonal stripes on the opposite wall. The orthogonal walls remained black. Animals were habituated to the environment for 3 × 30 min sessions before the onset of recordings. Recording sessions lasted for 60 min or until animals covered >70% of the environment, whichever occurred first. This led to recording sessions that were typically 30–40 min in duration. Animals were recorded once per day, and the electrodes were lowered by 25 μm at the conclusion of each recording. Data were screened offline for single units. Given the relatively small number of electrodes, typically fewer than three and often a single neuron were recorded in one session. The maximum number of simultaneously recorded neurons was 11. The environment was wiped down between animals with Nature’s Miracle Advanced Stain and Odor Eliminator. The recording environment itself was screened off from the rest of the room via a floor-to-ceiling black curtain that was closed during recordings. Tracking was done with an analog camera setup and then digitized with a positional sampling rate of 50 Hz and timestamped by the Axona recording system. LFPs were corecorded with single units, and were lowpass filtered from 0 to 500 Hz with a notch filter at 60 Hz. This channel was acquired at low resolution (250 Hz) and high resolution (4.8 kHz). The high-resolution trace was used for all spectral analyses.
Identification of firing fields. Spikes were converted into a binned rate map, which summed the number of spikes in each 2 cm x 2 cm bin. This rate map was then adaptively smoothed as in Skaggs et al. (1996). This smoothing procedure bins the recording environment into a 64 x 64 matrix, and the rate in each bin is determined by incrementally expanding a circle from each point until the number of spikes within the circle is greater than a fixed scaling parameter ($a$, set at 1 x 10$^6$ as in Skaggs et al., 1996) divided by the product of the squared occupancy ($N_{occ}$) and the radius of the circle ($r$) by the following equation:

$$N_{spikes} > \frac{\alpha}{N_{occ}r^2}$$

The firing rate in the bin is then the number of spikes divided by the number of samples multiplied by the sampling rate. The color map of each rate map in Figure 1 is determined by equally dividing the range of firing rate values in the bins of each map into steps on the color map, and selectivity produced from 100 shuffles of each cell in the dataset are illustrated in Figure 1E. The 99th percentile values were then determined from these distributions (sparsity = 0.976, information content = 0.0176, selectivity = 1.49) In addition, cells had to have at least one identified firing field (see firing field classification), and fields had to cover at least 2% but not >50% of the environment. This classification had the effect of removing putative interneurons from the analyses, which tended to have nondiscriminatory “fields” covering most of an environment.

Overdispersion of firing. Overdispersion was calculated as in Fenton et al. (2010), and is defined as the variance in a cell's firing rate for passes through a predefined place field. Place fields were defined as previously described, and only intervals containing trajectories through the locations defined as “firing field” were studied. Overdispersion was calculated as the SD in $z$ during each interval. The $z$ value was calculated as follows:

$$z = \frac{obs - exp}{\sqrt{exp}}$$

Where the observed number of spikes in each 5 s interval is defined as $obs$, and $exp$ is the expected number of spikes assuming pure Poisson firing, calculated as the product of time spent in a location during each interval, and the firing rate (spikes/time) in that location, for all of the locations visited during each 5 s epoch.

Network state estimation using corecorded neurons. We estimated network states from the spike train of pairs of corecorded cells at timescales corresponding to one theta oscillation (125 ms), one slow gamma oscillation (45 ms), and one fast gamma oscillation (25 ms). In order to estimate the correlation between pairs of cells, we used Kendall’s correlation as in Neymotin et al. (2017) and Talbot et al. (2010), since spike trains typically contain many co-occurring zero values, and Kendall’s correlation is robust against this.

Spectral analysis of theta rhythms. High-resolution LFP was bandpass filtered between 5 and 12 Hz, and then Fourier transformed. Instantaneous frequency and phase were determined by a Hilbert transform. To obtain estimates of the slope and the intercept of the relationship between running speed and frequency, a linear regression was performed on the running speed/frequency data. For the determination of spectral power in the theta band, a power spectrum of the high-resolution LFP trace was made using the pspectrum function in MATLAB 2019b with a frequency resolution of 0.5 Hz. Since spectral power can vary from animal to animal and depend on the duration of recording and the depth of the electrodes within the hippocampus, power was normalized within each recording such that the integral of the power between 3 and 30 Hz was 1 (as in Russell et al., 2006).

Spectral analysis of gamma rhythms and cross-frequency analysis. Analysis of gamma rhythms and cross-frequency coupling was modified from a previous technique (Colgin et al., 2009). High-resolution (4.8 kHz) LFP was transformed using a Morlet’s wavelet technique to determine the time-varying power in 2 Hz bins from 2 to 160 Hz. Spectral coherence between the original signal and the time-varying power was estimated using a magnitude squared function which consisted of a Hanning window FFT using 50% overlap. Briefly, this windowing procedure involved detecting an array of time points at which the power in the respective (fast or slow) gamma band exceeded 2 SDs from the average power in the fast or slow band; 160 ms of bandpass (slow or fast gamma, as appropriate) EEG centered on each of these time points was extracted, and the maximum and minimum gamma amplitudes in each of these were determined. These maxima and minima were used to eliminate overlapping segments; if an identified time point had an identical identified maximum and minimum power in the 160 ms window, it was excluded. Points were also excluded if they occurred within 100 ms of any other point. For the identified time points remaining, 400 ms sections of EEG centered on each time point were used for each individual gamma “window.” Individual gamma episodes were detected using the average time-varying power in the slow (25-50 Hz) and fast (65-140 Hz) bands. Gamma was detected when LFP epochs displayed power >2 SDs above the average power in each band across the recording. Gamma was then windowed as in Colgin et al. (2009). The relationship of these gamma episodes to the theta phase was determined using a Hilbert transform to derive instantaneous phase of theta, and this was then referenced to the time of maximum power for each.
Figure 1. The spatial properties of Ts65Dn place cells are normal. A, Twelve color-coded rate maps of place cells recorded from Ts65Dn (left column) and 2N Control littermate mice (right column). Place cells from both groups appeared normal, with well-defined firing fields. “Hotter” colors represent higher firing rates. A color bar beside each rate map represents the mean firing rate in both the maximum and minimum bins. Beside each cell is its spatial information content and spatial sparsity. The mean firing rate of each cell is shown above each. B, The information content of place cells is normal in Ts65Dn. C, The spatial sparsity of place cells is normal in Ts65Dn. D, The mean infield FR/Mean outfield FR is normal in Ts65Dn. E, The number of shuffles for spatial information content, spatial sparsity, and spatial selectivity are normal in Ts65Dn.
gamma epoch. Duplicated gamma episodes caused by overlapping time windows were removed by discarding gamma episodes with equal maxima, and requiring that each subtype of gamma be temporally separated by at least 100 ms.

Spectral analysis of sharp-waves. High-resolution (4.8 kHz) LFP was filtered using a Morlet's wavelet centered on 200 Hz with a bandwidth of 50 Hz, giving a filter band of 150-250 Hz. Detection of sharp waves on the resultant filtered signal was determined similarly to what was done previously (Gillespie et al., 2016); epochs of filtered LFP were determined to be sharp waves if they were >3 SDs above the amplitude of the filtered signal, and that this magnitude persisted for 15 ms or longer.

Histology and determination of electrode position. At the conclusion of experiments, animals were deeply anesthetized with isoflurane, and then were injected with an overdose of sodium pentobarbitone intraperitoneally. Animals were then perfused transcardially with 0.9% PBS, followed by 9% formalin. The brains were decapitated, and their brains were removed and placed into a cylindrical container holding a solution of 9% formalin for 24-48 h. Brains were then transferred to a 30% sucrose solution, where they were kept until they sunk from the top of the container to the bottom. Brains were removed from solution, quickly frozen, and then sectioned coronally into slices of 40 μm thickness. Slices were mounted, and then Nissl-stained to visualize cell bodies and make the electrode positions apparent. The track made by the tetrodes and its end position was determined through microscopic examination of the slides, and the depth at which each cell was recorded was determined by back-calculation from this final location.

Results
Since a central aspect of a “disruption” view of hippocampal dysfunction suggests that the spatial properties of hippocampal place cells should be abnormal, we first sought to examine the spatial firing properties of place cells in Ts65Dn and 2N control animals. Single units recorded from Ts65Dn animal 2N littersmates were isolated as animals explored a 40 × 40 cm black polycarbonate arena. A total of 179 neurons were isolated in the Ts65Dn group, and 72 from the 2N Control group. These neurons were manually separated into putative interneurons and pyramidal cells on the basis of their location specificity, firing rate, and waveform. After removing all interneurons, there were n = 119 neurons remaining in the Ts65Dn group (mouse n = 4) and n = 63 (mouse n = 4) neurons remaining in the 2N control group.

The spatial properties of Ts65Dn place cells are not different from control
Noninterneuron cells recorded from the hippocampus of 2N and Ts65Dn animals were classified as place-encoding if they passed the thresholds for the standard place-cell metrics information content, sparsity, and selectivity generated by the temporal shuffling of spikes (see Materials and Methods). In addition, cells were included only if they had identified fields covering not <2% of the environment and not >50% of the environment in total (see Materials and Methods). This left n = 107 cells in the Ts65Dn group and n = 42 cells in the 2N control group. Significantly more hippocampal cells sampled from Ts65Dn animals passed the thresholds to be considered “place” cells than cells sampled from 2N Control animals (Ts65Dn = 107 of 119; 89.9%, 2N Control = 42 of 64; 65.6%, \( \chi^2 = 16.23, p < 0.001 \)). However, place cells in both 2N and Ts65Dn groups appeared superficially similar (Fig. 1A). There were no differences between 2N and Ts65Dn cells in spatial information content (Fig. 1B; mean information content ± SEM [bits/spike], Ts65Dn = 0.269 ± 0.033; 2N Control = 0.258 ± 0.065, Z = 1.323, p = 0.186, not significant), spatial sparsity (Fig. 1C; mean sparsity ± SEM, Ts65Dn = 0.744 ± 0.017; 2N Control = 0.772 ± 0.028, Z = 1.314, p = 0.189, not significant), or ratio of infield to outfield firing rates (Fig. 1D; mean infield/outfield firing rate ratio ± SEM, Ts65Dn = 3.03 ± 0.16, 2N Control = 3.03 ± 0.35, Z = 0.437, p = 0.662, not significant). Place field size was similar between the groups of cells (mean % place field coverage ± SEM; Ts65Dn = 14.37 ± 1.09, 2N Control = 12.78 ± 1.65, Z = 1.07, p = 0.287, not significant). However, Ts65Dn place cells fired significantly fewer spikes in bursts of spikes (<10 ms interspike interval) than control (mean spikes in bursts/single spikes ± SEM, Ts65Dn = 0.581 ± 0.116, 2N Control = 0.612 ± 0.102, Z = 2.44, p = 0.0146).

The frequency of hippocampal theta rhythm is higher in Ts65Dn animals than 2N control
Sessions from which single units were recorded were used for the analysis of hippocampal theta rhythm (Ts65Dn, sessions = 21, mice = 4 [2 mice, 4 sessions; 1 mouse 5 sessions, 1 mouse, 8 sessions]; 2N Control, sessions = 13, mice = 4 [3 mice = 3 sessions, 1 mouse = 4 sessions]). Both Ts65Dn and 2N Control animals displayed a typical dominant theta rhythm in their hippocampal LFP (Fig. 2A). The mean frequency of theta rhythm in Ts65Dn animals was significantly higher than 2N control (Fig. 2A,D; mean frequency [Hz] ± SEM, Ts65Dn = 8.42 ± 0.029 Hz, 2N Control = 8.25 ± 0.040 Hz, t = 3.54, p = 0.001). While theta frequency is linearly correlated with running speed (McFarland et al., 1975), the difference in frequency between Ts65Dn and 2N control theta frequency was not because of difference in running speed. The Ts65Dn animals did run faster on average than 2N Control (Fig. 2E; mean speed ± SEM [cm/s], Ts65Dn = 14.21 ± 0.614 cm/s, 2N Control = 8.96 ± 0.355 cm/s, Z = 4.64, p = 3.44 \( 10^{-6} \)), but the intercept of a linear regression through the speed/frequency data was still greater for Ts65Dn animals compared with control (Fig. 2B; theta intercept ± SEM [Hz], Ts65Dn = 8.349 ± 0.040 Hz, 2N Control = 8.115 ± 0.067 Hz, t = 3.22, p = 0.007). There was an overrepresentation of data from one animal in the Ts65Dn group that contributed 8 of 21 sessions. We therefore considered theta intercept as a per-animal average as well as a per-session value. Theta intercept remained higher in the Ts65Dn group when the unit of comparison was per-animal rather than per-session despite the attendant smaller n (Fig. 2F; mean intercept ± SEM [Hz], Ts65Dn = 8.37 ± 0.07 Hz, 2N Control = 8.16 ± 0.05, t = 2.49, p = 0.047). The slope of the linear relationship between theta frequency and running speed appeared to be flatter in Ts65Dn animals than control, but this apparent difference did not reach significance (mean slope ± SEM [Hz/cm/s], Ts65Dn = 0.0063 ± 0.0012, 2N Control = 0.0084 ± 0.0029, t = −0.768, p = 0.448, not significant). Restricting analyses only to regimens in which the animals ran at the most similar speeds (Fig. 2H,I), Ts65Dn theta frequency was higher than 2N control at speeds from 20 to 40 cm/s (mean frequency ± SEM [Hz], Ts65Dn = 8.77 ± 0.05, 2N Control = 8.44 ± 0.08, t = 3.58, p = 0.0011) and at the most similar narrow regimen of speeds between 9-11 cm/s (mean frequency ± SEM [Hz], Ts65Dn = 8.73 ± 0.05, 2N Control = 8.51 ± 0.09, t = 2.47, p = 0.019). While there was no overall difference in the mean normalized power over the entire theta band between Ts65Dn and 2N control animals.
Figure 2. The frequency of hippocampal LFP theta rhythm is higher in Ts65Dn animals than 2N control. A, Spectrograms represent the spectral power between 0 and 40 Hz over four individual 600 s recording sessions (two Ts65Dn, left, green; two 2N control, blue, right). “Hotter” colors represent higher power. The approximate location of the theta band (5-11 Hz) is illustrated for each spectrogram with a red bar above the band. A strong band of high power in the theta range is evident in each recording, typical for recordings from hippocampus. B, The intercept
Fig. 2D), this was because of a right-shifted preferred band of theta at higher frequencies in the Ts65Dn animals. The peak power of theta was higher in Ts65Dn animals than 2N control (Fig. 2D; normalized peak theta power ± SEM; Ts65Dn = 0.012 ± 7.66E-4, 2N control = 0.009 ± 5.16E-4, t = 2.464, p = 0.0193).

Ts65Dn hippocampal cells are more likely to be theta rhythm, and are highly phase-locked to hippocampal theta rhythm compared with 2N control

Although there was no difference in the spatial characteristics of place cells in Ts65Dn animals compared with control, cells in these animals were much more tightly phase-locked to the ongoing LFP theta than control (Fig. 3A,C,D). The phasic directional vector was much larger in Ts65Dn cells (n = 119) than 2N Control (n = 63, mean vector length ± SEM; Ts65Dn = 0.143 ± 0.007, 2N Control = 0.083 ± 0.009, Z = 5.054, p = 4.319E-7), demonstrating that spikes most often occurred on regular phases of theta in Ts65Dn cells. In contrast, the firing of 2N control cells was distributed normally through phases of theta as the animal moved through the firing field of each cell. Ts65Dn cells tended to lock to the peak or trough of theta (Fig. 5B,D,E,F); the direction of the mean vector of these cells was significantly clustered compared with 2N control (F1,180) = 38.75, p = 3.3E-6). The mean phase direction of 2N control cells was evenly distributed at all phases of theta (U = 146.2, p = 0.100). In contrast, the phase directions of the directional vector of Ts65Dn cells were highly clustered (U = 160.1, p = 0.001).

Determination of the intrinsic rhythmity of the spiking of cells in both groups was conducted using the maximum likelihood estimation method as in Climer et al. (2015) This method derives a rhythmic modulation index ranging from 0 (no rhythmicity) to 1 (completely rhythmic). Cells recorded from Ts65Dn animals had significantly higher rhythmity indices than cells recorded from 2N Control animals (Fig. 4A–C) (rhythmity index ± SEM; Ts65Dn = 0.487 ± 0.025, 2N Control = 0.320 ± 0.037, Z = 3.83, p = 1.26E-4). These data indicate a much greater degree of intrinsic theta rhythmity in the cells of Ts65Dn animals. The maximum likelihood estimation method also determines whether the rhythmic fit is a significantly better fit to the spiking data than a flat, nonrhythmic fit. Ts65Dn cells were much more likely to be better fit by a rhythmic fit than a flat decay than were 2N Control cells (Fig. 4D; percent significantly rhythmic; Ts65Dn = 56.94%, 2N Control = 26.15%, C2 = 12.27, p = 0.0005).

The spike trains of corecorded cells suggest a stronger network state in Ts65Dn animals

In addition to the abnormally strong spike/phase relationship of single units to theta in the Ts65Dn animals, we investigated the co-firing relationship between pairs of corecorded hippocampal cells, as in Talbot et al. (2018) at various timescales to estimate the strength of the overall network state. We recorded 178 cell pairs from Ts65Dn animals, and 50 cell pairs from 2N Control animals at a resolution similar to fast gamma (25 ms), slow gamma (45 ms), and theta (125 ms). Spike trains were more likely to be more highly correlated in Ts65Dn than 2N control animals at 25 ms (Fig. 5G; two-sample Kolmogorov–Smirnov test; D = 0.25, p = 0.0271), 45 ms (Fig. 5B; D = 0.2454, p = 0.0309), and 125 ms (Fig. 5A; D = 0.2331, p = 0.0432) resolution. While the correlation between spike trains generally decreased over recordings, we found that the correlation between spike trains was more stable for Ts65Dn neurons between halves of a recording session compared with 2N Control at 45 ms and 125 ms resolutions (Fig. 5G–I; change in Kendall’s tau ± SEM; 45 ms, Ts65Dn = −0.014 ± 0.011, 2N = −0.062 ± 0.017, D = 0.280, p = 0.0163; 125 ms, Ts65Dn = −0.19 × 10−4 ± 0.006, 2N = −0.026 ± 0.009, D = 0.326, p = 0.0011), but not at the lowest (25 ms) resolution corresponding approximately to fast gamma (change in Kendall’s tau ± SEM, Ts65Dn = −0.028 ± 0.015, 2N = −0.105 ± 0.025, D = 0.251, p = 0.056, not significant). We also examined the variability of firing rates of place cells as animals moved through each cell’s place field (Fig. 5D; overdispersion), as in Fenton et al. (2010), but found no difference in firing rate variability between Ts65Dn and 2N Control place cells (overdispersion, Z ± SEM; Ts65Dn = 3.083 ± 0.180, 2N Control = 3.129 ± 0.234, Z = 0.538, p = 0.591, not significant).

Increased coherence and abnormal phasic relationships between theta and gamma rhythms in Ts65Dn animals

Spectral coherence between theta and gamma bands (Fig. 6A,B) overall was greater in the Ts65Dn animals than the 2N controls (Fig. 6C–F; mean theta/gamma coherence ± SEM; Ts65Dn = 0.032 ± 0.007, 2N control = 0.016 ± 0.002, t = 2.07, p = 0.044). The peak of coherence between theta and fast gamma appeared to occur at higher frequencies in Ts65Dn animals than control (Fig. 6C; peak coherence ± SEM; Ts65Dn = 75.52 ± 2.77 Hz, 2N Control = 71.54 ± 0.79 Hz). Subtraction of the mean cross-frequency coherence in the theta band of Ts65Dn animals from 2N control demonstrated greater coherence between both fast and slow gamma and higher frequencies of theta in the Ts65Dn animals (Fig. 6D). However, when the coherence for each recording session was normalized within each session, there was relatively greater coherence between both gamma bands and lower frequency theta in 2N control animals (Fig. 6D).

Previous work has shown a phasic relationship between the theta wave and the different bands of gamma, with gamma typically occurring at predictable phases of, and appearing to be superimposed on, theta oscillations (Colgin et al., 2009; Bieri et al., 2014; Schomburg et al., 2014). To examine the segregation of gamma on the theta wave in more detail, we bandpass filtered the EEG 3 times into theta, slow gamma, and fast gamma bands, and then divided theta-pass EEG into peak-peak epochs. We then counted the number of times that either slow or fast gamma
epochs in the relevant bandpassed EEG occurred on the same theta wave. In both the Ts65Dn (35.75 ± 1.71%) and 2N control (36.92 ± 1.59%), fast and slow gamma occurred on the same theta wave (~35%) of the maximum possible coincidences, with no difference in co-occurrence because of genotype (t = −0.466, p = 0.645, not significant; Fig. 6B). The location of the superposition of fast and slow gamma on theta is typically segregated, with fast gamma occurring close to the peak of theta (Fig. 6G; Rayleigh test, Z = 6.59, r = 8.057−4), whereas fast gamma occurs on the falling phase of theta nearer to the trough (Rayleigh test, Z = 3.99, p = 0.016; Fig. 6H). However, in Ts65Dn animals, there is markedly less phasic separation. While the mean theta phase of slow gamma in 2N control animals is unipolar and relatively specific to the rising phase of theta, the mean theta phase of Ts65Dn slow gamma is relatively evenly distributed (Fig. 6G; Rayleigh test, Z = 0.69, r = 0.505, not significant), as is the mean theta phase of fast gamma (Fig. 6H; Rayleigh test, Z = 0.135, r = 0.877, not significant). While the mean theta phase of slow gamma was not different between 2N control and Ts65Dn animals (Watson–Williams
test, $F_{(1,36)} = 3.23$, $p = 0.081$, not significant), fast gamma in Ts65Dn animals often occurred at opposite phases of theta than 2N control (Watson-Williams test, $F_{(1,36)} = 4.34$, $p = 0.044$).

Previous research has demonstrated that neurons within the hippocampus are sometimes phase-locked to gamma oscillations (Colgin et al., 2009; Carr et al., 2012; Bieri et al., 2014). In both 2N and Ts65Dn animals, we find that, on average, cells are phase-entrained to both slow and fast gamma (Fig. 5I–N). However, spikes fired by neurons in 2N control animals were equally likely to be phase-locked to slow and fast gamma (14 of 61 phase-locked to slow gamma, 10 of 61 phase-locked to fast gamma, $Q = 0.830$, $p = 0.36$) while Ts65Dn neurons were more likely to be phase-locked to slow gamma compared with fast (13 of 114 fast, 25 of 114 slow, $Q = 4.55$, $p = 0.033$).

**Discussion**

The Ts65Dn mouse model of DS is known to exhibit learning and memory deficits that are central to the syndrome (Reeves et al., 1995), strongly implying hippocampal dysfunction. In vitro experiments have demonstrated a range of abnormal morphologic and electrophysiological differences in the hippocampus of these animals (Siarey et al., 1997; Kurt et al., 2004; Costa and Grybko, 2005; Hernández-González et al., 2015; Aldred et al., 2018). In these experiments, however, we observe hippocampal place cells that do not obviously differ from control animals, similar to what has been seen in other model systems, such as the Fragile-X Fmr-1 mutant (Talbot et al., 2018). Unlike the Fmr-1
model, however, we observe in the Ts65Dn mouse a somewhat stronger hippocampal network state when examining the co fir ing relationship between neurons. Examination of the relationship of single units to theta clarifies the probable cause of this difference. Ts65Dn neurons are hyper phase-associated to theta, and are themselves dramatically more intrinsically theta rhythmic. This hyperorganization of phase strongly implies excess inhibitory control, since control of timing is highly dependent on GABAergic inputs (Mann and Paulsen, 2007). We also observe that almost all the neurons sampled from Ts65Dn animals meet the criteria to be considered "place" cells, while a more expected proportion of 2N control cells sampled met this threshold. Again, this observation points to a hyper-rigid organization of hippocampal activity in the Ts65Dn animal.

The question of which interneuron population is responsible for these changes in the Ts65Dn animal remains open. Parvalbumin-positive interneurons are involved in local theta generation, whereas somatostatin-positive neurons are responsible for entraining longer-range inputs from entorhinal cortex (Amilhon et al., 2015). Consistent with the abnormal intrinsic rhythmicity and abnormal long-range phase relationships in the Ts65Dn model, it seems likely that both populations are affected. It has been previously shown that the somatostatin-positive Martinotti cells are more strongly connected with excitatory pyramidal cells in the hippocampus of Ts65Dn animals, whereas the parvalbumin interneurons become hyperexcitable (Zorrilla de San Martin et al., 2020).

Segregation of firing of single units on the theta wave is thought to gate information flow into epochs of encoding and retrieval based on the synchronicity between regions at specific points on the hippocampal theta cycle (Hasselmo et al., 2002; Manns et al., 2007). While it may seem initially paradoxical that

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**Figure 5.** Stronger network states in the Ts65Dn hippocampus. **A-C,** Cumulative probability that a pair of corecorded place cells have spike trains of a given correlation at 125 ms (A), 45 ms (B), and 25 ms (C) resolution. Gray dashed line indicates the point of no correlation. **D,** The overdispersion of firing in the place fields of Ts65Dn (green) and 2N (blue) animals. Half-violin represents the distribution of values. Red line indicates the mean of each group. **E, F,** Kendall’s tau of each corecorded cell pair in the Ts65Dn (n = 144 pairs) and 2N control (n = 33 pairs) in the first and second halves of each recording session. **G-I,** Probability of a given difference in Kendall’s tau between the first and second halves of a recording session. Red dashed line indicates no change in tau.
Figure 6. Coherence between hippocampal theta and gamma rhythms is altered in Ts65Dn mice. A, Example cross-frequency coherence spectrograph showing the coherence between frequency bands. In this example, spectral energy in the theta band is highly coherent with energy in the fast gamma band, and there is moderate coherence between theta and slow gamma. White dashed rectangle represents the location of the theta phasic band. B, Cross-frequency coherence spectrograms for two 2N control animals (left pair) and two Ts65Dn animals (right pair). The cross-frequency spectrograms are restricted to the theta band for clarity. C, The mean cross-spectral coherence (solid lines) ± SEM (shaded regions) between theta and other frequencies. D, Top, The mean cross-frequency coherence over all recordings in the 2N control group subtracted from the mean cross-frequency coherence over all recordings in the Ts65Dn group. Bottom,
stronger network states and increased theta might lead to poorer hippocampal function, it seems likely that the stronger network states are an epiphenomenon of the abnormal phase-locking of place cells in the Ts65Dn animal, and it does not represent more effective information processing. While it has been shown that weak network states in the hippocampus are associated with poor function, we believe this is the first demonstration that hyperorganization among neurons in the hippocampus can lead to similar deficits.

Long-range inputs from entorhinal cortex to hippocampus via gamma oscillations are critical for successful encoding and retrieval, and we see a recapitulation of the previously observed segregation of fast and slow gamma onto different phases of the theta cycle in 2N control animals. Interestingly, there is increased coherence between theta and gamma overall in the Ts65Dn animals, again seemingly in contradiction to the known behavioral differences suggesting hippocampal deficits in Ts65Dn. However, just as with theta rhythm itself, the phasic relationship between theta and fast gamma is disturbed in these animals. While slow gamma occurred at similar theta phases to control animals, fast gamma was as likely to occur at the peak of theta as at the trough. This failure to segregate information switching from CA3 to MEC (and from retrospective to prospective coding) may underlie the learning deficits stronger network states and increased theta might lead to poorer hippocampal function, it seems likely that the stronger network states are an epiphenomenon of the abnormal phase-locking of place cells in the Ts65Dn animal, and it does not represent more effective information processing. While it has been shown that weak network states in the hippocampus are associated with poor function, we believe this is the first demonstration that hyperorganization among neurons in the hippocampus can lead to similar deficits.

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seen in both Ts65Dn animals, and humans with DS. It is important to note, however, that although we examine slow and fast gamma in a similar manner and using an established technique (Colgin et al., 2009; Igarashi et al., 2014) we do not resolve gamma into other, overlapping “mid-gamma” frequencies that have been identified by other authors.

The observations that GABA antagonism and the reduction of specific GABA receptor subunits (Rueda et al., 2008; Vidal et al., 2018) can correct some of the cognitive consequences of DS suggest that inhibitory control of hippocampus is a central feature (Bezaire et al., 2016; Huh et al., 2016). Although modeling work suggests that theta within CA1 can be produced without direct septal input, septal input is critically involved in regulating phasic relationships with theta (Mysin et al., 2019). An imbalance in the ratio of inhibitory GABAergic to excitatory cholinergic/glutamatergic projections from MS to HPC in Ts65Dn animals may be sufficient to perturb phasic relationships with theta within the hippocampus.

Given recent data suggesting that artificially prolonged sharp-wave ripples correlate with better spatial memory (Fernández-Ruiz et al., 2019), we were initially puzzled that Ts65Dn animals displayed ripples that were dramatically longer, on average, than 2N controls. However, it is critical to consider that it is not ripple activity per se that is typically linked with memory consolidation, but instead it is the replay of place cell sequences during these brief epochs, as well as the reverse replay of sequences observed during these events during sleep. Identification of meaningful sequences typically requires the simultaneous recording of tens of neurons as animals follow a predictable course on a linear shuttle track, or the recording of hundreds of neurons during exploration of a 2D environment. The relatively low number of simultaneously recorded neurons in the present study prevents close analysis of this question, and investigating ripple activity in the ensemble during awake exploration and again during sleep remains a promising object for further study, even more so in light of the known abnormalities in sleep architecture seen in the Ts65Dn mouse (Colas et al., 2008) and DS individuals (Bassell et al., 2015; Fernandez et al., 2017; Lukowski et al., 2020).

Although we are unable to make conclusions regarding sequence activity differences between 2N control and Ts65Dn animals, longer-duration intrinsically generated sharp-wave ripples are typically correlated with increased demands on memory; that is, longer-duration ripples indicate that the memory system is working harder (Fernández-Ruiz et al., 2019). In these experiments, animals had no special task demands, and the sharp-wave ripples of 2N control animals were predictably short. The longer-duration ripples of Ts65Dn animals may illustrate their relatively worse memory performance generally; indeed, environmental novelty is associated with longer-duration ripples (Fernández-Ruiz et al., 2019). This may suggest that the environment was perpetually novel to the Ts65Dn animals, while the 2N control animals rapidly learned the spatial configuration of the recording chamber. In support of this notion, it has been demonstrated that Ts65Dn animals perform poorly on tests of novel object recognition at 24 h delays (Colas et al., 2013). Future studies should broaden the spectrum of behavioral tests beyond the novel object recognition task to determine whether the failure to become familiar with spatial configurations is a general deficit observed in Ts65Dn animals. Also of interest is determining whether longer-duration ripples are phenomenologically or mechanistically linked with poor encoding of novelty by artificially shortening ripples by optogenetic silencing and examining the effect on behavior in a spatial memory task. Likewise, future studies could determine whether pharmacological treatment with GABA antagonists (that improve memory performance) have the effect of shortening the duration of ripples in this model.

In these experiments, we find electrophysiological evidence of perturbation in hippocampal synchrony in the Ts65Dn mouse model of DS that are consistent with the learning and memory deficits seen in this model. As in many other perturbation experiments, the gross appearance of place cells in these animals is normal, but the central changes are in the phasic relationship between place cells and theta rhythm, and between theta and other types of oscillation in LFP. In the Ts65Dn model, and in contrast to other similar models such as Fmr-1, we observe that the system becomes hyperorganized. The phasic relationship of single units becomes more rigid, and their co-firing relationships become more similar. This constellation of changes matches the hypothesis that the critical deficit in DS is excess inhibition. However, whether this excess inhibition arises locally from excess interneuronal contact with pyramidal neurons, or via an altered excitatory/inhibitory balance in projections to or from, remains to be discovered.

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