A distinctive subpopulation of medial septal slow-firing neurons promote hippocampal activation and theta oscillations

Running title: Pro-arousal slow-firing neurons in MSvDB

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Abstract

The medial septum - vertical limb of the diagonal band of Broca (MSvDB) is important for normal hippocampal functions and theta oscillations. While many previous studies have focused on understanding how MSVDB neurons fire rhythmic bursts to pace hippocampal theta oscillations, a significant portion of MSVDB neurons are slow-firing and thus do not pace theta oscillations. The function of these MSVDB neurons, especially their role in modulating hippocampal activity, remains unknown. We recorded MSVDB neuronal ensembles in behaving rats, and identified a distinct, physiologically homogeneous subpopulation of slow-firing neurons (overall firing <4Hz) which shared three features: 1) much higher firing rate during rapid-eye-movement (REM) sleep than during slow-wave (SW) sleep; 2) temporary activation associated with transient arousals during SW sleep; 3) brief responses (15~30 ms latency) to auditory stimuli. Analysis of the fine temporal relationship of their spiking and theta oscillations showed that unlike the theta-pacing neurons, the firing of these pro-arousal neurons follows theta oscillations. However, their activity precedes short-term increases in hippocampal oscillation power in the theta and gamma range lasting for a few seconds. Together, these results suggest that these pro-arousal slow-firing MSvDB neurons may function collectively to promote hippocampal activation.
**Introduction**

The medial septum - vertical limb of the diagonal band of Broca (MSvDB) heavily innervates the hippocampus through cholinergic, GABAergic, and glutamatergic projections (Gritti et al. 2006; Kiss et al. 1990; Kohler et al. 1984; Lewis et al. 1967). These projections are essential for normal hippocampal functions. Lesion or inactivation of MSvDB recapitulates the memory deficit resulting from hippocampal lesions and eliminates hippocampal theta oscillations (Gray and McNaughton 1983; Green and Arduini 1954; Mahut 1972; Mizumori et al. 1990; Winson 1978).

To achieve a mechanistic understanding of the critical role of MSvDB in hippocampal functioning, it is essential to elucidate how the neuronal activity in MSvDB modulates hippocampal activity, in particular its dynamic interactions on fine temporal scales. Indeed, this issue has been studied extensively at the electrophysiological level over the last few decades. Those studies have largely focused on the fast-firing, rhythmic-bursting MSvDB neurons, and demonstrated that these cells are the pacemaker of hippocampal theta oscillations (Ford et al. 1989; Hangya et al. 2009; Morales et al. 1971; Petsche et al. 1962).

However, in parallel with their heterogeneity in terms of neurotransmitters, the physiological properties of MSvDB neurons are similarly diverse. Besides the extensively studied fast-firing, rhythmic-bursting neurons, many MSvDB neurons are nonetheless slow-firing and do not fire theta-range rhythmic bursts (Ford et al. 1989; Gaztelu and Buno 1982; Stewart and Fox 1989). As such, these latter cells are unlikely candidates to participate in pacing theta oscillations. The function of
these slow-firing neurons remains unknown, partly because these slow-firing MSvDB neurons have received little attention in the literature. However, a recent study demonstrated for the first time that neurochemically identified MSvDB cholinergic neurons are slow-firing and do not pace theta oscillations (Simon et al. 2006). Given the well-established role of MSvDB cholinergic neurons in normal hippocampal theta oscillations and hippocampal-dependent learning and memory (representative reviews: Hasselmo 2006; Jerusalinsky et al. 1997; Vanderwolf 1988), this new finding calls for further investigation of the functions of the slow-firing MSvDB neurons.

Therefore the first goal of the present current study was to comprehensively characterize the physiological properties of slow-firing MSvDB neurons in different behavioral contexts in freely-moving rats. More importantly, in order to provide new insights on how the slow-firing MSvDB neurons modulate hippocampal activity, we aimed at determining how their neuronal activity is correlated with changes in hippocampal oscillatory activities, including theta oscillations. By using chronically-implanted multi-electrode arrays (MEA) in freely-moving rats to record simultaneously neuronal ensembles in MSvDB and local field potentials (LFPs) in the hippocampus, we first investigated the firing properties of MSvDB slow-firing neurons during the sleep-waking cycle and during exposure to auditory stimuli. Based on neurophysiological properties measured during three distinct behavioral contexts, we identified a functionally homogenous MSvDB subpopulation. We then investigated how the firing of these neurons is correlated with the oscillatory activities in the hippocampus, and
demonstrated that the activity of these slow-firing MSvDB neurons precedes hippocampal activations for a few seconds. Our results therefore provide new insights on how a functionally-identified subpopulation of slow-firing MSvDB neurons may dynamically enhance hippocampal activity on the time scale of a few seconds.

**Materials and Methods**

**Animals**

Animal use and procedures were approved by the Duke IACUC and performed in accordance with NIH guidelines. Eight adult male Long-Evans rats (300-500g) were used in the experiments.

**Electrodes and surgery**

The multi-electrode assembly or array (MEA) was constructed similarly to earlier studies (Lin and Nicolelis 2008; Nicolelis et al. 2003). On each assembly, two 29-gauge stainless-steel cannulae were secured, with tips separated by 0.8mm horizontally (anterior-posterior) and 0.5 mm vertically, targeting anterior (deeper) and posterior (shallower) parts of the MSvDB. As a bundle, 8 or 16 35-μm tungsten wire electrodes were threaded together through each cannula. The bundles could be advanced by microdrives with precision over the course of the experiments. The total range of advancement was 2 mm in length. Wires spread slightly from the cannula tip after being pushed out, covering a semi cone-shaped area for about 1-1.5 mm in diameter at the end (Figure 1A). Since the implant
was slightly angled (below), the semi cone-shaped spreading would start from mid-line, then advance with a slight preference towards one hemisphere (Figure 1A). Ipsilateral to this preferred hemisphere, the hippocampal multi-electrode array was arranged in a 4x4 form (250 μm spacing) for hippocampal LFP recordings. Two of the four rows, aimed at dentate gyrus (DG), were 0.7mm longer than the rest, which aimed at CA1.

Surgeries for electrode implantation have been described earlier (Nicolelis et al. 1997). Animals were anesthetized with ketamine (100mg/kg) and xylazine (5mg/kg) and positioned in a stereotaxic frame. Atropine (0.02mg) was used to reduce airway secretion. Stainless steel screws were secured above frontal cortex and cerebellum, serving as grounds. Craniotomies were opened above the MSvDB region (AP +1.5- 0 mm, ML 0.5-1.5 mm relative to Bregma ) and hippocampus unilaterally (AP -4.0mm, ML 2.5mm, ~1x1 mm) (Paxinos and Watson 2005). The MEA with two movable electrode bundles was lowered into MSvDB with an angle of 9.5° on the coronal plane, targeting two locations in MSvDB: AP+1.1/+0.3mm, ML 0mm, -6.2/-5.7mm below dura. A 4x4 array was lowered into one hemisphere to record from CA1 (-2.2mm below dura) and dentate gyrus (DG, -2.9mm below dura). Dental acrylic was used to cover and secure the implant with the help of anchoring screws. Rats were allowed to recover for at least 14 days after surgery before recordings.

Undisturbed sleep-waking behavior
Animal behavior was monitored on-line with a camera and recorded on videotape. Rats were allowed to freely move around or sleep for 1~1.5 hr in the recording session, in the light/sound attenuated chamber to which they had been habituated to and the same one in which they would perform the behavioral task. To minimize disturbance from the experimenter, sleep-waking states were monitored remotely using an online detection algorithm based on LFP spectral features (Gervasoni et al. 2004).

**Behavioral task and auditory stimuli**

In each recording session, well-trained rats performed a variant of the Go/NoGo behavioral task for 1~1.5 hr. The apparatus (Med Associates Inc, VT), behavioral training and task details have been described elsewhere (Lin and Nicolelis 2008). Three cues, an 80dB 6kHz tone, an 80dB white noise sound or a light (on), each lasting 2 sec, were presented in a random order during the task, with inter-trial interval (ISI) randomly varied between 6-18 sec (6, 8,10,14,18 sec). Each animal was assigned to respond to one of the cues within 5 sec of the cue onset to get reward (~ 0.04mL 10% sucrose). After they retrieved their reward, or if they did not respond, a new trial (new ITI) would start. If they licked during the ITI, the trial would be reset. There was no punishment for responding to the other two cues, or for not responding to the rewarding cue. For each type of auditory stimulus, it could be either a rewarding cue, a distracter when the other auditory cue was associated with reward, or non-relevant when the light cue was associated with reward.
Electrophysiological recording

Electrical signals were amplified with TBSI 2x or 1x headstages (Triangle BioSystems, Inc. Durham, NC, USA). Neuronal and local field potential (LFP) signals were filtered at 154-8.8kHz and 0.4-240Hz, digitized at 40kHz and 2kHz, respectively, and recorded with a Multichannel Acquisition Processor (Plexon Inc, Dallas, TX). MSvDB electrode bundles were advanced at 60 or 125 μm steps between recording sessions to minimize sampling from overlapping neuronal populations. Single units were identified online based on spike waveform to aid off-line processing of single unit data (see next section). LFP signals were also transmitted online to a remote computer to monitor the sleep-waking states of the animal.

Data analysis

Single unit isolation (spike sorting)

Spike waveforms were processed with an Offline Sorter (Plexon Inc, Dallas, TX) to obtain single units. Effort was used to ensure the isolation of single units, with signal-to-noise ratio larger than 3 and minimal amount (<0.1%) of “spike collision” (spikes occurred within the action potential refractory period, set as 1.2ms) based on the interspike interval histogram (Nicolelis et al. 2003). The timestamps of the single units and LFP signals were further analyzed in Matlab (The MathWorks, Natick, MA).
Sleep-waking analysis and SIA definition

Sleep states were characterized as described earlier (Gervasoni et al. 2004). Spectral features were obtained to determine the behavioral state of the animal as three gross categories, waking (WK), slow-wave (SW) sleep and rapid-eye-movement (REM) sleep. Firing rates of individual neurons were calculated for the whole recording session (over-all or average firing rate) and individual sleep-waking states (Figure 1A). Instantaneous firing rates were calculated by binning spikes into each second and smoothed for ±4 sec around each bin.

Small-amplitude irregular activity (SIA) epochs were further determined in the SW sleep episodes, based on methods described elsewhere (Jarosiewicz and Skaggs 2004). Briefly, root-mean-square amplitude of hippocampal LFP was calculated and smoothed for 0.5 sec bins. An amplitude threshold was set at a local minimum in the amplitude distribution around the 20th percentile of the distribution (Figure 2A). Individual SIA epochs were defined as a continuous period with 80% of the amplitude below amplitude threshold, and no gaps (large amplitude LFP) longer than 1.5 sec. The SIA onset was initially identified as amplitude trace crossing the threshold (large to small), and then slightly adjusted for 1 bin / 0.5 sec at most (most often no adjustment) to find the sharpest drop of amplitude. Minimal duration of SIA was set to 1.5 sec. With the choice of amplitude threshold, about 20% of the SW sleep was identified as SIA, in agreement with earlier studies (Jarosiewicz et al. 2002). The remaining part of the SW sleep was defined as large-amplitude irregular activity (LIA) epochs.
Firing property indices for individual neurons

The REM index for each neuron was defined as the ratio between firing rates of REM and SW sleep.

Response to auditory stimuli was characterized using peristimulus time histograms (PSTH) with 5 ms bins. The auditory response index (Aud index) was defined as the ratio between averaged PSTH values during 15-35ms after and 300-5 ms before the stimulus onset (Figure 3B). Unless specified, the Aud index was calculated for the white noise stimulus. Aud index > 1 represents excitatory response, while index <1 represents inhibitory ones.

Histograms similar to PSTH were calculated for individual neurons, with spikes aligned to SIA onset at 100 ms bins (Figure 2B). Z-scores of firing associated with SIA onset were calculated, defined as the difference between averaged PSTH values during -1 ~ + 0.5 sec around SIA onset, and baseline time -4 ~ -1.5 sec, divided by the standard deviation of PSTH values during the baseline time. The ratio between averaged PSTH values around SIA onset and the baseline was also calculated. The SIA index was subsequently defined as the product of the z-score and the ratio. These two components incorporate the substantial firing changes associated with SIA onset in two types of neurons we observed: one with extremely low firing during baseline (LIA), and a sudden firing at SIA onset (high ratio, but low Z-score due to high variance caused by the sporadic firing during baseline); another with relatively stable firing during baseline, and a relatively small (low ratio) but very significant increase of firing at SIA onset (high Z-score). SIA index > 1 represents an excitatory association,
while index < -1 suggests inhibition. SIA indices between -1 and 1 suggest no clear firing rate changes, and were treated as 0 for log transformed value. Except for these values, log transform of SIA index was performed on the absolute value, with original signs assigned to the log value (SIA index >1 became Log index > 0; SIA index < -1 became Log index < 0).

Histograms of log transformed indices were plotted and fitted with the local fit function (Dr. Partha Mitra and www.chronux.org). The local minima between the two modes were used as threshold values. Principle component analysis on the three indices was performed for all the slow-firing neurons, and the first principle component was extracted to aggregate the indices.

K-means clustering, a standard algorithm in the MatLab program, was used to cluster neurons based on the log transformed indices. This algorithm partitions data points into a defined number of clusters, and with iteration, minimizes the summed Euclidean distance to cluster centeroids.

**Theta related analysis**

One of the DG LFPs was filtered between 4.5-9Hz for theta oscillations and theta phases were calculated using Hilbert transform on the filtered signal. The rhythmicity index was calculated based on auto-correlogram in REM sleep (Jobert et al. 1989; Kitchigina et al. 2003). Phase of individual spikes was calculated as the Hilbert phase at the time of the spike. Phase preference of each neuron was displayed with phase distributions (histograms). Phase-locking strength (Z values) and p-values were calculated with circular statistics (Rayleigh
test, MatLab toolbox from Berens and Velasco 2009). The Z-shift method to analyze the fine temporal relationship between phase-locked neuronal activity and LFPtheta oscillations was adopted from earlier studies (Hangya et al. 2009; Siapas et al. 2005). For the Z-shift analysis, one LFP from MSvDB was also used to quantify local theta oscillations in MSvDB. Neuronal spikes were shifted temporally relative to LFPs, to calculate phase-locking Z values (Figure 6A). The temporal shift that results in maximal Z values indicates the temporal relationship between the neuronal activity and the microstructures of the LFP theta oscillations. A positive spike shift suggests spikes leading LFP theta, and vice versa.

Cross-correlation analysis

Cross-correlation functions were calculated for neuronal pairs, with spikes from the whole recording session, or selected states during the recording session, such as waking, SW or REM sleep. Cross-correlation functions were normalized by firing rates (over-all or during specific states) of the two neurons, and the length of recording or state. The long tails of the function ([-20 -10] and [10 20] sec) were used as an empirical baseline for individual pairs (Lin et al. 2006), during which the neuronal firing is supposedly not correlated. The average baseline values were subtracted out, and the fluctuations during the baseline were used to estimate the significant cross-correlation between -10 and 10 seconds. One-way ANOVA with Bonferroni-corrected multiple comparisons was used for analysis on cross-correlation values. To estimate the width of the cross-
correlation functions, average cross-correlation value within the window of [-1 1] sec was calculated. If this value was larger than baseline fluctuations (mean+3xs.d.), the two temporal points at which the smoothed cross-correlation function dropped to half of the average value were determined, and the time window between these two points was deemed as the half-width of the cross-correlation function.

Spectral analysis

Spectral analyses were performed with the Chronux package based on multitaper spectral methods (Dr. Partha Mitra and www.chronux.org). For different frequency ranges, slightly different moving windows were used: 0-20Hz, 2 sec window with 0.5 sec moving steps; 30-300Hz, 0.5 sec / 0.25 sec. To calculate spike-triggered or event-triggered spectrograms, spectrograms around individual spikes/events were aligned and averaged. For plots during the waking state, spikes occurring [-1 +1] sec around behavioral events (cues, licks etc) were excluded to prevent potential confounding factors. Spike-triggered spectrograms were normalized by the baseline period [-8 -5] sec before the spike. Z-scores for each spectrogram were calculated based on the fluctuations during the baseline. Individual time-frequency pixels in the spectrogram with Z-scores larger than a pre-determined Z value (5.7, 8.1 for the two frequency ranges, square-root of the number of time bins) were counted as significant pixels. The number of these significant pixels was counted in Figure 8B.
Results

We recorded from 300 MSvDB single units in freely-moving and behaving animals (n = 8 rats). In the following report, we focus mostly on the slow-firing neurons with an average firing rate below 4Hz (n=190) (4Hz demarcation defined by Simon et al. 2006).

Physiological properties of slow-firing MSvDB neurons in three behavioral contexts

To comprehensively characterize the physiological properties of these slow-firing neurons, we investigated their activity in three distinct behavioral contexts which are known to modulate MSvDB neuronal activity: (1) different sleep states; (2) neuronal activity associated with transient arousal epochs during sleep; and (3) neuronal responses to auditory stimulus.

First, we investigated the sleep-waking state-dependent firing property of the slow-firing MSvDB neurons. Similar to the rhythmic-bursting neurons (Morales et al. 1971; Simon et al. 2006; Sweeney et al. 1992), the firing rates of the entire population of slow-firing MSvDB neurons are lowest during slow-wave (SW) sleep (0.76±1.14 Hz, mean±s.d.), intermediate during waking (WK) (1.14±1.17 Hz) and highest during rapid-eye-movement (REM) sleep (2.98±2.35 Hz). Figure 1B shows the state-dependent firing rates for a representative neuron and normalized firing rates for the population (Friedman Test, p<0.001 for all three states; and p<0.001 between each two states, Wilcoxon signed rank test).
To understand how uniform such state-dependent firing rate fluctuations manifest on the single neuron level, we compared the firing rates of individual MSvDB neurons in REM to SW (Figure 1C) and WK to REM (Figure 1D). The scatter plots indicate that not all neurons have the firing rate modulation similar to that shown in the population. To quantify the state-dependent firing of individual neurons, especially the propensity to fire more frequently during REM than during SW, we devised a REM index for each neuron. This REM index is obtained as the ratio between the neuron’s firing rates during REM and SW. The distribution of REM index was roughly bimodal, with a subset of MSvDB neurons showing very high REM-indices (Figure 1E). Slightly more than half (52%, 98/190) of the slow-firing neurons were categorized as REM+ (see Methods for the selection of threshold).

Second, we investigated the detailed firing properties of MSvDB neurons during SW sleep when the average firing rate is the lowest among the major arousal states. Particularly, we identified transient arousal epochs, the small-amplitude irregular activity (SIA) epochs (Figure 2A) (Jarosiewicz et al. 2002; Jarosiewicz and Skaggs 2004), and studied how these transient states were associated with the neuronal activities in the MSvDB. A subset of MSvDB neurons increased their activity starting around 1 to 0.5 sec before SIA onsets (example in Figure 2B, population results in Figure 2C), suggesting that the activity of these neurons may promote the transition into SIAs. Compared to their firing rates during SIA epochs, these neurons fired much less frequently during the slow-wave epochs LIA (large-amplitude irregular activity), which accounts for
most of the SW sleep (data not shown). To quantify the transient firing around SIA onsets, an SIA index was calculated for each MSvDB neuron (Figure 2D, see Methods for detail). Overall, 52% (99/190) of MSvDB neurons showed a significant firing rate increase around the SIA onset, and were categorized as SIA+ (see Methods for the selection of threshold).

Third, we investigated the firing properties of slow-firing MSvDB neurons in response to auditory stimuli (2 sec white noise) in a reward-related behavioral context. Consistent with previous reports (Miller and Freedman 1993), we found a subset of MSvDB neurons with prominent auditory responses, in the form of very transient firing (single spikes or short bursts) at 15~35ms after stimulus onset (see example in Figure 3A). Such an auditory-evoked neuronal response was sensory-related but not reward-related, because the response magnitudes co-varied with sound intensity levels (data not shown) but did not depend on whether or not the sound was associated with the prediction of reward (Figure 3D). To characterize this auditory response, we devised an auditory response index (Aud index, see Methods for detail). Similar to the distributions of REM index and the SIA index, the Aud index distribution was also bimodal (Figure 3C). More than half (57%, 109/190) of the MSvDB neurons were categorized as Aud+ (Figure 3C, see Methods for the selection of threshold).

Multiple firing features uniquely define a distinctive population of slow-firing MSvDB neurons

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Interestingly, most of the neurons that were REM+ and SIA+ were also Aud+ (71 out of 81, 87.7%), while the majority (72 out of 109, 66%) of the remaining neurons were Aud- (Figure 3C), suggesting that these properties which we have investigated may be tightly related. To systematically investigate the relationship between the three physiological properties of MSvDB slow-firing neurons, we first calculated the correlation between any two of the three indices. Strong correlations were found for all three pairs of indices, when all the slow-firing neurons were pooled together (Figure 4C, all p<0.001, significance tested with non-parametric Spearman rank correlation; correlation coefficients, r= 0.55, 0.59, 0.51, for pairs of indices).

Using the three indices to construct a 3-D plot (Figure 4A), we observed that the MSvDB neurons bearing all three properties (REM+, SIA+, Aud+) formed a cluster separated from the remaining neurons, indicating the existence of two groups of slow-firing neurons (REM+/SIA+/Aud+ and the remaining neurons). To confirm this observation, we used an un-supervised k-means clustering algorithm on all the MSvDB slow-firing neurons to separate them into two clusters based on these three indices. The result matched very well with our two groups (REM+/SIA+/Aud+, 82% overlapping with cluster 1) (Figure 4A). In fact, similar results can be achieved when clustering with any two of these indices (data not shown). Very similar separation can also be seen if the first principal component (PC) of the 3-D plot was used to aggregate the three indices into one dimension (PC1, the first principal component), which showed a bimodal distribution matching the two groups (Figure 4B).
Furthermore, the correlations between indices within either group were not significant (Figure 4C, except between REM index and Aud index for the remaining neurons), corroborating the notion that two separate and homogenous subpopulations of neurons were present. All these results suggest that the REM+/SIA+/Aud+ neurons form a distinctive MSvDB subpopulation that is physiologically homogeneous (Figure 4D). Since these firing properties are associated with behavioral or electrophysiological arousal, we will refer to this subpopulation as pro-arousal slow-firing neurons, and the remaining slow-firing neurons as other slow-firing neurons.

Fine temporal relationship of MSvDB pro-arousal slow-firing neurons with theta oscillations

To provide context to compare our pro-arousal slow-firing neurons with earlier studies examining theta-related properties of MSvDB neurons, we characterized their theta-related firing modulations during REM sleep, which has the highest level of theta oscillations and engages rhythmic firing of MSvDB neurons the most (Sweeney et al. 1992). Particularly, we characterized the rhythmic firing in the theta frequency range, phase-locking to theta oscillations, and inter-spike interval (ISI) distributions of MSvDB neurons.

Figure 5A-D shows neuronal activities during one REM episode, for one pro-arousal slow-firing, one other slow-firing, and one fast-firing rhythmic-bursting neuron. The theta-related analysis shown in Figure 5 was based entirely on hippocampal (DG) LFP. Despite the high levels of theta oscillations in the REM
episode (Figure 5A), pro-arousal slow-firing neurons showed little rhythmic firing in the theta frequency range compared to the other two types of neurons, visualized as the theta-related side bands in the auto-correlation function (Figure 5D and 5H). In general, it is known that many non-rhythmic neurons can phase-lock to theta oscillations (e.g. Type II neurons defined in King et al. 1998). Even though not rhythmic, most of the pro-arousal slow-firing neurons were phase-locked to theta oscillations (Figure 5B and 5E), with a lower level of phase preference compared to the two groups (Figure 5F). Interestingly, for the neurons which were consistently phase-locked to theta oscillations, the pro-arousal neurons, but not the other two groups of neurons, preferentially fired at the falling phase of the local theta oscillations in MS (data not shown). Consistent with the phase-locking, their ISI distributions showed a prominent peak in the theta interval (100-180 msec) (Figure 5C and 5G). These results suggest that pro-arousal slow-firing neurons have weaker, but consistent theta-related firing compared to other slow-firing and the fast-firing populations.

In order to further understand the relationship between the firing of MSvDB neurons and the pacing of theta oscillations, we investigated the fine temporal structure of MSvDB spike timing relative to the microstructure of theta oscillations using Z-shift analysis (Figure 6A-B), which has been used to infer the causal relationship between neuronal spiking activity and theta oscillations (Siapas et al. 2005). Among the neurons that were significantly phase-locked to theta oscillations (phase-locking p<0.01; 84%, 81% and 94% of pro-arousal slow-firing, other slow firing and fast-firing neurons), the spike timing of most pro-
arousal slow-firing neurons was best correlated with local theta oscillations in the MSvDB that occurred 10-20 msec prior to their spiking. Such results suggest that pro-arousal slow-firing neurons are likely followers of theta paces in the MSvDB. In contrast, the spike timing of most other slow-firing and fast-firing MSvDB neurons consistently leads the microstructure of theta oscillations by 10-40 msec (Figure 6C). Overall, our analysis on the millisecond-level spike timing indicates that the spiking of pro-arousal slow-firing neurons is likely driven, as least partially, by local theta oscillations.

**MSvDB pro-arousal slow-firing neurons fire together and promote hippocampal activation in a state-dependent manner**

To further understand how MSvDB neurons operate in relation to each other, we investigated their propensity to fire together, using cross-correlation analyses, on pro-arousal slow-firing, other slow-firing and fast-firing MSvDB neurons. As shown in the example in Figure 7A and the population results in Figure 7B-C, almost all pairs of pro-arousal slow-firing neurons showed significant positive correlation, indicating that these neurons are more likely to fire action potentials when another pro-arousal slow-firing neuron fires within a few seconds (Figure 7B). However, this propensity was much reduced between other types of MSvDB neurons and between pro-arousal slow-firing neurons and other neuronal types (Figure 7B). Moreover, almost none of the pro-arousal slow-firing neuron pairs showed anti-correlated firing (negative peak in cross-correlations), while 10-20% of other pair combinations were significantly anti-correlated (Figure
7B). This pattern was consistently observed in all behavioral states, although the width of correlation functions (positive correlations) differed among states (cross-correlation half-width, WK: 3.2±1.3 sec, SW, 0.2±1.3 sec, REM, 3.8±2.8 sec; REM>WK>SW, Wilcoxon signed ranks test between each pair). The strength of correlated firing, as determined by the average amplitude of cross-correlation functions, was significantly higher between pro-arousal slow-firing neurons than between other neuronal pairs (Figure 7C). These results indicate that pro-arousal slow-firing neurons fire together within a few seconds of each other in all behavioral states, and suggest that these cells may operate as a coherent neuronal ensemble.

Finally and most importantly, in order to better understand how MSvDB neurons modulate large-scale oscillatory activity patterns in the hippocampus, we investigated how the activity of individual MSvDB neurons are correlated with simultaneously recorded hippocampal LFP activity, by analyzing spike-triggered spectrograms. Single neuron (Figure 8A) and population data (Figure 8B) show that during REM and WK, the firing of pro-arousal slow-firing neurons was associated with increased theta oscillations (7-10Hz) and its harmonics. The peak of theta power increase occurred, on average, after the timing of the spike. This suggests that the firing of pro-arousal slow-firing neurons promotes the increase of theta oscillation power. Increases in the power of high gamma oscillations (70~130Hz) was also observed. These increases lasted about 2 sec. In addition, the firing of pro-arousal slow-firing neurons also preceded a decrease in the power for oscillations below 6Hz. In contrast, other slow-firing neurons
were not associated with such changes. During SW sleep which does not favor theta oscillations, the firing of pro-arousal slow-firing neurons was associated with a decrease of power in the 0-20 Hz oscillatory range for several seconds. Such a decrease in low frequency power corresponds to the diminished LFP amplitude during the SIA epochs.

Overall, these results suggest that, in contrast to the precise spike timing of pro-arousal slow-firing neurons being influenced by individual theta cycles on a millisecond level (Figure 5-6), the firing of pro-arousal slow-firing neurons promotes an increase in theta and gamma oscillation power during theta-dominant WK and REM states, and a decrease in low frequency power and the onset of SIA during SW sleep. This pattern of modulation was not associated with the spiking of other slow-firing neurons. It is important to note that the modulations of power (but not phase) of hippocampal oscillatory patterns operate on the time scale of a few seconds, consistent with the time scale of the collective behavior of the pro-arousal slow-firing neurons (cross-correlation results in Figure 7).

Discussion

Unlike previous MSvDB studies which most often focus on fast-firing, rhythmic bursting neurons and theta-pacing, the current study was designed to investigate the neurophysiological properties of the slow-firing MSvDB neurons. We successfully identified a homogeneous subpopulation of slow-firing neurons, comprehensively characterized their physiological properties and investigated
how their activity may modulate large-scale hippocampal activity patterns.

Specifically, we demonstrated that a subpopulation of slow-firing MSvDB neurons can be uniquely defined and separated from other MSvDB neurons by three highly correlated features (Figure 4): higher firing rate during REM sleep (Figure 1), firing rate increase before transient arousals during SW sleep (Figure 2) and short-latency transient responses to auditory stimuli at 15-35 ms (Figure 3).

These pro-arousal slow-firing neurons were also distinct in their intrinsic firing properties compared to other MSvDB neurons: non-rhythmic firing, less-prominent theta phase-locking, and likely followers of theta oscillations rather than theta pace-makers (Figure 5-6). Furthermore, they fire together as a group (Figure 7) and their firing precedes and likely promotes an increase in power of theta and gamma oscillations during waking and REM sleep, as well as suppresses slow-waves while promoting SIA epochs during SW sleep (Figure 8).

Our data suggest that these pro-arousal slow-firing neurons may act as a coherent neuronal ensemble in vivo to collectively promote the activation of hippocampal networks in all behavioral states. These results represent one of the few studies that delineate how a functionally distinctive group of neurons in a subcortical neuromodulatory system influence the activity of its cortical target.

**A distinctive, putatively cholinergic subpopulation of neurons in MSvDB**

Previous studies on MSvDB neuronal activities have mostly focused on fast-firing neurons as well as disparate physiological properties in isolated domains, e.g. firing rate modulations in all major arousal states (Morales et al. 1971;
Sweeney et al. 1992), responses to behavioral events (Mercer and Remley 1979; Zin et al. 1977) and intrinsic firing properties in relation to theta oscillations (King et al. 1998; Macadar et al. 1970; Petsche et al. 1962). The lack of comprehensive characterization across those feature domains has made it difficult to compare and integrate results collected in different studies. Our study provides for the first time a comprehensive characterization of slow-firing MSvDB neurons in normal behaving animals and shows that many disparate features are in fact highly related (Figure 4). Most importantly, the convergence of these features uniquely defines a group of homogeneous MSvDB neurons that are distinct from other MSvDB neurons.

The constellation of these features suggests that this homogeneous group of neurons likely shares the same neurochemical identity, in particular cholinergic. Multiple lines of supporting evidence indicate that the properties of the pro-arousal slow-firing neurons remarkably resemble the known physiological attributes of the MSvDB cholinergic neurons. First, the overall low firing rates were consistent with the findings from neurochemically identified MSvDB cholinergic neurons (Simon et al. 2006). Second, their firing rate modulations are consistent with the fluctuations in hippocampal ACh concentration during sleep-waking cycles (Bianchi et al. 2003; Marrosu et al. 1995). Third, their firing precedes and may promote transient arousal epochs during SW sleep, where a nicotinic cholinergic mechanism has been shown as a contributing mechanism (Lena et al. 2004). Fourth, a previous study suggested that neurons with the transient response to auditory stimulus might be cholinergic (Miller and
Therefore, we propose that the pro-arousal slow-firing MSvDB neurons that we identified here are putative MSvDB cholinergic neurons. Although converging evidence supports the view that these are cholinergic neurons, difficulties in determining the exact neurochemical identity of chronically recorded neurons in behaving animals have prevented us from definitively confirming their cholinergic identity. Future experiments employing juxtacellular labeling or optogenetic tools (Zhang et al. 2007) will likely resolve this issue. In fact, our comprehensive characterization points out a promising direction for identifying MSvDB cholinergic neurons, and will likely reduce the otherwise tremendous effort required in these studies.

Relationship between MSvDB pro-arousal slow-firing neurons and theta oscillations

Previous studies have presented conflicting views on the roles of different types of MSvDB neurons in pacing and generating hippocampal theta activity (Apartis et al. 1998; Brazhnik and Fox 1997; Hangya et al. 2009; Markram and Segal 1990; Simon et al. 2006; Sotty et al. 2003). Our large-scale survey of MSvDB neurons indicates that other slow-firing and fast-firing neurons are more tightly phase-locked with theta oscillations, compared to pro-arousal slow-firing neurons. The former two groups also lead theta oscillations by tens of milliseconds, supporting their roles in pacing theta oscillations (Figure 6). On the contrary, pro-arousal slow-firing neurons are only mildly phase-locked (Figure 5), and follow, rather than lead, theta oscillations (Figure 6). In fact, the pro-arousal
neurons may be theta followers regardless of the behavioral context of the theta oscillations – their spiking lack behind both REM and WK theta (Figure 6), and their firing rates during theta in REM or Type I theta in WK (particularly, active exploration of objects) were not different statistically (data not shown).

Despite following local theta oscillations at the millisecond level, our results indicate that the firing of pro-arousal slow-firing neurons likely modulates hippocampal oscillatory power, lasting for several seconds (Figure 8). Particularly, during WK and REM states, in which theta oscillations are favored, these neurons likely promote the increase in power of the higher-frequency theta / gamma oscillations in the hippocampus. Therefore, our results suggest that the pro-arousal slow-firing neurons are not involved in the cycle-by-cycle pacing of hippocampal theta oscillations. Rather, they are entrained to and recruited by theta oscillations, and their firing in turn may sustain and enhance the power of ongoing hippocampal theta oscillations. Taking into account that the rhythmic bursting neurons pace the frequency of theta oscillations (Hangya et al. 2009), we suggest that the frequency and amplitude (power) of hippocampal theta oscillations may be dynamically modulated by separate populations of MSvDB neurons.

In spite of the wealth of literature on the pharmacology and lesion of cholinergic actions (Dougherty et al. 1998; Lee et al. 1994; Parent and Baxter 2004; Steckler et al. 1995), surprisingly little is known about how the firing of MSvDB cholinergic neurons is related to, and modulates, in turn, the activity of hippocampal networks in normal behaving animals. This is largely due to the lack
of knowledge about how cholinergic neurons behave \textit{in vivo}. If this unique
subpopulation we identified is cholinergic, our results suggest that MSvDB
cholinergic neurons do not pace theta oscillations. Rather, their precise spike
timing is influenced by the preceding theta cycle in the local circuit, and their
firing in turn promotes theta oscillation power, lasting for a few seconds. Our
investigation therefore provides new insights on theta-related properties of
MSvDB cholinergic neurons and a novel mechanistic hypothesis on how they
modulate hippocampal activity, particularly theta oscillation amplitude. These
conclusions are also consistent with our recent finding in urethane-anesthetized
rats that hippocampal ACh level and the activity of the putative MSvDB
cholinergic neurons lag behind, and therefore do not participate in, the fast onset
and pacing of theta oscillations (Zhang et al. 2010).

**New insights on the function of MSvDB pro-arousal slow-firing neurons**

As discussed above, one function of the pro-arousal slow-firing neurons
may be to promote a power increase of theta / gamma oscillations and to
suppress low frequency oscillations (0-6Hz) in the hippocampus during WK and
REM states.

On the other hand, in behavioral states in which theta oscillations are not
favored, such as SW sleep, the firing of the pro-arousal slow-firing neurons likely
suppresses low frequency oscillations (0-20Hz). In line with our observation that
they increased their activity just before SIA onsets (Figure 2), another function of
these neurons may be to promote the onset of SIA epochs during SW sleep.
While the neural mechanisms responsible for generating the transient arousal state SIA are largely unknown (Jackson et al. 2008; Jarosiewicz et al. 2002), it has been shown that the cholinergic system plays an important role - knock-out mice without the β2 subunit of nicotinic receptors showed decreased frequency of SIA occurrence (Lena et al. 2004). This finding not only supports our proposal that the specific neuronal population we identified is likely cholinergic neurons, but also suggests that MSvDB cholinergic neurons may be a crucial component of the SIA generation mechanism. Furthermore, these pro-arousal neurons may also be generally associated with transitions to behavioral arousal or electrophysiologically activated states, as they gradually increased their firing rates starting from 2-3 seconds before the transitions from SW sleep to WK or from intermediate sleep (IS, also called transition to paradoxical sleep, t-PS) to REM sleep, and transiently increased firing in the one second before SW to IS transition (data not shown).

Together, their strong association with SIA onsets, tight coupling with an increase in theta/gamma oscillation power and decrease in low frequency oscillation power suggest that MSvDB pro-arousal slow-firing neurons may act as a generalized mechanism for hippocampal activation and electrophysiological arousal. Direct evidence beyond the temporal relationship between spike timing and power changes warrants future investigations to confirm this hypothesis. Beyond the above-discussed association and potential promotion of electrophysiological activation of the hippocampus, by naming these neurons "pro-arousal" we are not suggesting that their activity could directly produce
behavioral arousal or awakening. Behavioral arousal is controlled by more caudal nuclei (for example, reviewed in Jones 2008), and the MSvDB pro-arousal neurons may be an important node to relay such arousal to their target areas, i.e. hippocampus and associated areas.

The mechanism by which the pro-arousal neurons potentially produce hippocampal activation is not known. Direct projection from these pro-arousal neurons to the hippocampus may be a parsimonious mechanism. However, septohippocampal projection is said to have a rough topographic organization, with different groups of neurons preferentially innervating different part of the hippocampus along the longitudinal axis (Amaral and Kurz 1985; Segal and Landis 1974), while LFPs along the longitudinal axis may not be entirely synchronized. Our current techniques limit our capability to identify whether the recorded MSvDB neurons project directly to the dorsal hippocampus where we recorded the LFPs. This issue, and the activation mechanism need to be investigated in future studies.

Last but not least, if this subpopulation is indeed cholinergic, this putative cholinergic network not only participates in the wake-sleep states modulation, but is also activated in behavioral context such as responding to external stimuli. In behaving animals, the short-lasting increase of theta power caused by the firing of these neurons may serve as short-term tags (Vertes 2005) and a favorable network state for synaptic plasticity (Larson et al. 1986; Pavlides et al. 1988). The consequent release of ACh in the hippocampus may, in parallel, act on muscarinic receptors to produce relatively long-lasting effects on promoting
signaling pathways that lead to activation of kinases (Giovannini et al. 2005) and immediate-early genes (IEGs) (Wirtshafter 2005). Through these concerted actions, the activity of MSvDB cholinergic neurons can modulate both the short-term activity and long-term plastic changes of the hippocampal network, thus fulfilling the role of the cholinergic system in hippocampal synaptic plasticity and learning and memory.

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References


Gritti I, Henny P, Galloni F, Mainville L, Mariotti M, and Jones BE. Stereological estimates of the basal forebrain cell population in the rat, including neurons containing choline acetyltransferase, glutamic acid decarboxylase or phosphate-activated glutaminase and colocalizing vesicular glutamate transporters. Neuroscience 143: 1051-1064, 2006.


Figure Legends

Figure 1. Slow-firing neurons have higher firing rate in REM sleep.

A. Schematic and examples of the recording site demonstrated with histology and immunohistochemistry. Left, the cannula implant design and estimated target area (blue area) in MSvDB. Blue dotted rectangle, area in Middle. Middle, histology (Nissl staining) image. The empty space (arrow) resulted from cannula implantation. Arrow head indicates initial spreading of the bundled electrodes from the tip of the cannula. Electrodes advanced progressively for another 1 mm during which the recordings were obtained. Scale bar on top left corner, 200μm. Orange dotted rectangle, area in Right. Right, ChAT staining demonstrating cholinergic neurons in MSvDB, dispersed in the area being recorded. Scale bar on top left corner, 200μm. B. Firing rates of a representative (upper) and all slow-firing MSvDB neurons (lower) change across waking (WK), SW and REM sleep. Box plot of firing rates (lower) in different behavioral states normalized to average firing rate of each individual neuron. Dotted line indicating 1. For each box plot, the central mark (middle of notch) is the median; box edges mark the 25th and 75th percentiles; red crosses are outliers; the whiskers extend to the most extreme data points not including outliers; non-overlapping notches between groups indicate a significant difference between medians (significance level = 0.05). Firing rate was highest in REM, lowest in SW, and intermediate in WK (#, p<0.001, Wilcoxon signed rank test). C-D. 2-D plots of firing rate comparison for individual neurons, between REM and SW (C), REM and WK (D). Black, pro-arousal slow-firing neurons
defined in Figure 4; blue, other slow-firing neurons; blue lines indicate unity. 

Histogram of REM index for individual neurons. Inset, histogram of log-transformed index values with local fitting of distribution curve (black). Green dotted line, local minimum used as the cutoff threshold (REM index=8.0).

Neurons were therefore grouped into REM- (92/190) and REM+ (98/190) groups.

**Figure 2. Many slow-firing neurons increase firing around SIA onset.**

A. Left, example of SIA and LIA epochs during SW sleep. Blue, LFP in DG; red, smoothed root-mean-square amplitude of LFP (0.5sec bins); magenta and cyan lines below indicate identified SIA and LIA epochs. Middle, histogram of amplitude distributions for the whole recording session. Red dotted line, amplitude threshold used to define SIA epochs (see Materials and Methods). Right, histogram of SIA duration in the same recording session. B. Example of transient firing around SIA onset. Left and middle panels, raster plots of spikes (black) around SIA or LIA onset (aligned, onset time is 0). Magenta or blue dots, beginning and end of individual SIA or LIA epochs. Epochs sorted according to length (display cut-off is 10 sec). Right panels, averaged firing rate around the onset of SIA or LIA. C. Pseudocolored PSTHs (time 0, SIA onset) of all slow-firing MSvDB neurons, sorted according to the SIA index. Firing rates normalized to average firing rates of individual neurons during SW sleep. Two gray bars below x-axis indicates the peak (-1~+0.5 sec) and baseline (-4 ~ -1.5 sec) periods used to calculate SIA index. D. Histogram of SIA index for all slow-firing neurons. Inset, histogram of log-transformed index values with local fitting of
distribution curve (black). Green dotted line, local minimum used as the cutoff
threshold (SIA index=5.0). Neurons were grouped as SIA- (91/190) or SIA+
(99/190).

Figure 3. Most REM+/SIA+ neurons have transient auditory responses.

A. Example of a neuron with transient auditory response. Gray bar on the
top indicates a 2 sec auditory stimulus (an 80Hz white noise sound). Raster plot
in the middle shows the trial-by-trial firing this neuron (black dot, single spike),
aligned to the onset of the auditory stimuli (time = 0). Peristimulus histogram
(PSTH) in the bottom summarizes the response of this neuron, showing a very
strong but transient response, tightly locked at 15-30ms after the stimulus onset.

B. Pseudocolored PSTHs of all slow-firing MSvDB neurons, sorted according to
the auditory response index (Aud index, see methods). The example in A is # 53
on this plot (black arrow). Two gray bars below indicate the peak (15~35ms) and
baseline (-300 ~ -5 ms) periods used to calculate Aud index. C. Histogram of Aud
index for two groups of neurons, REM+/SIA+ (red) and the remaining (blue).

Most REM+/SIA+ neurons had higher Aud index, while the majority of the
remaining neurons had low Aud index. Right, histogram of log-transformed index
values of all slow-firing neurons with local fitting of distribution curve (black).

Green dotted line, local minimum used as the cutoff threshold (Aud index=4.5).

Neurons were grouped as Aud- (81/190) or Aud+ (109/190). D. Responses to
auditory cue (white noise) not depending on the role of the cue. Pseudocolored
PSTHs of all slow-firing MSvDB neurons, categorized based on the role of the
white noise cue for individual animals. White lines in the middle separate pro-arousal slow-firing neurons (upper panels) and other slow-firing neurons (lower panels). See Figure 4 for definition of pro-arousal and other slow-firing neurons.

Figure 4. Multiple firing features uniquely define a distinctive population of slow-firing MSvDB neurons.

A. REM+/SIA+/Aud+ neurons formed a distinctive population. Three-dimensional plots of the three log-transformed indices. Circles indicate clustering results from unsupervised k-means algorithm. The two clusters were very consistent with the division based on firing indices, suggesting that REM+/SIA+/Aud+ neurons are distinctive from the remaining neurons. B. Aggregation of the three indices into the first principle component (PC1) shows similar separation of the two groups of neurons. C. Correlation between indices on 2-D views of plots in A. Black and blue lines, correlation calculated in the respective group; red lines, correlation for all neurons. Solid lines indicate significant correlation (all red lines, and the blue line in left panel, p<0.001; significance tested with non-parametric Spearman rank correlation); dotted lines indicate no significant correlation. D. Diagram showing the distribution of neurons among the REM+/-, Aud+/- and SIA+/- groups. Neuron numbers of other individual categories are shown in blue.

Figure 5. Theta-related firing properties of pro-arousal slow-firing neurons in REM sleep.
A-D. Example of theta-related properties of three types of neurons recorded in the same REM episode. **A. Upper**, spectrogram of hippocampal LFP during the REM episode. **Lower**, smoothed instantaneous firing rates. **B. Phase preferences** of the three individual neurons. The pro-arousal slow-firing neuron (proA) had weaker phase modulation. **C. Interspike interval (ISI, sec)** distribution of the three neurons. **D. Autocorrelation** during REM episode. The pro-arousal slow-firing neuron was not rhythmic-firing, while the other slow-firing neuron and the fast-firing neuron had characteristic rhythmic side peaks in their autocorrelation functions. Autocorrelation functions normalized to their individual maximal values. **E. Population plots of phase-locking** during REM sleep of the three groups of neurons. Units sorted according to phase-locking strength (Rayleigh test critical value Z). Non-significant (p>0.05) neurons were displayed as null in the bottom. Pseudocolor indicates phase distribution (%). **F. Sorted phase-locking strength** (Z values) for the three groups in E. Vertical line indicates significant phase-locking (to the right of the line, p<0.05). Among phase-locked neurons, Z values were generally small for the pro-arousal slow-firing neurons, compared to the other two groups. **G. ISI distribution** for the three groups. Pro-arousal slow-firing neurons had a clear peak around 0.1-0.18 sec. **H. Population plots of autocorrelation** (pseudocolor, normalized to maximum of individual correlation). Units sorted according to rhythmicity index. Many fast-firing neurons were rhythmic firing, unlike most pro-arousal slow-firing neurons.

**Figure 6. Pro-arousal slow-firing follow local theta oscillations in MSvDB.**
**A.** Schematics of the Z-shift method. Blue trace, LFP; black and red rasters, original and time-shifted neuronal spikes. Phase-locking Z values are calculated while shifting neuronal spikes relative to LFP signals. Positive shift in time, if results in larger Z value, suggests that the neuronal spikes are better phase-locked to LFP signals in the future. **B.** An example of Z values from one pro-arousal slow-firing neuron, calculated for theta oscillations for both WK and REM episodes concatenated together. HP_LFP, LFP theta oscillations in the hippocampus; MS_LFP, LFP theta in MSvDB. The maximal Z value for phase-locking to theta oscillations in MSvDB occurred before 0, suggesting that the neurons spikes followed the theta oscillations in MSvDB LFP in the past. **C.** Histogram of time-shift distribution corresponding to maximal Z values of significantly phase-locked (p<0.01) MSvDB neurons during WK, REM sleep, or both states. Positive spike shift suggests spike leading LFP theta, and vice versa. Pro-arousal slow-firing neurons mostly follow LFP theta in MSvDB, while fast-firing neurons mostly lead theta oscillations both in the hippocampus and MSvDB.

**Figure 7. Pro-arousal slow-firing neurons fire together.**

**A.** Example of cross-correlation between neuronal pairs. Panels showing cross-correlation in each recording session as a whole (ALL), or cross-correlation broken-down by states (WK: waking, SW: slow-wave sleep, REM: REM-sleep). Three types of neurons are shown: pro-arousal slow-firing (proA), other slow-firing (Other) and fast-firing (Fast) neurons. For every two panels, the left panel shows cross-correlation of a pair of neurons from the same type, and the right
panel shows cross-correlation of a pair of neurons from two different types. Cross-correlation between two pro-arousal slow-firing neurons is more prominent than those from other pairs, in all behavioral states. B. Proportion (average±SEM) of significant cross-correlation of all neuronal pairs at different time lags. Positive values depicting original cross-correlations above the upper significant threshold (mean + 3 x s.d.); negative values depicting the ones below the lower significant threshold (mean - 3 x s.d.). The proportion of positively significant cross-correlation is higher for pro-arousal slow-firing pairs than other types of pairs. No pro-arousal slow-firing pairs showed negative cross-correlations. Color scheme the same as in A. C. Average cross-correlation values (average±SEM) of all neuronal pairs for different time windows. Cross-correlation between two pro-arousal slow-firing neurons is stronger than other types of pairs. Color scheme the same as in A. All comparisons tested in one-way ANOVA with Bonferroni-corrected multiple comparisons. Significance levels are labeled only when value of the pair from two pro-arousal slow-firing neurons is significantly larger than any other types of pairs. Δ, p<0.05; +, p<0.01; #, p<0.001.

**Figure 8. Pro-arousal slow-firing cholinergic neurons promote hippocampal activation.**

A. Example of averaged spectrograms around spikes of a pro-arousal slow-firing and an other slow-firing neuron recorded in the same session. Low and high frequency ranges (0-20, 30-300Hz) were displayed separately.
Spectrograms were aligned to the spikes of individual neurons (time 0, black vertical line in the center). Black horizontal bar on lower left indicates the baseline period. For normalization, average log power during the baseline period at each frequency was subtracted, and pseudocolors indicate the power increase or decrease. During REM and WK, the firing of pro-arousal slow-firing neurons promoted increase of theta power (7-10Hz, and 2x harmonics), and in some neurons (this example) the power in high gamma range. Their firing was also associated to decrease in power in frequency bands below 6Hz. In SW sleep, the firing of these neurons was associated with a marked decrease of power in low frequency range (0-20Hz). Firing of other slow-firing neuron did not lead to comparable spectral changes. B. Significance plots for spectrograms of all pro-arousal slow-firing and other slow-firing neurons. Significance based on Z-scores calculated with baseline (see methods). Heat-plots indicate the percentage of neurons beyond Z-score threshold, for positive (left two columns, warm color) and negative (right two columns, cool color) Z-scores, corresponding to power increase and decrease. The pattern is similar to individual example.
Pro-arousal

Other slow-firing neurons

REM index = \( \frac{FR_{REM}}{FR_{SWS}} \)
Figure 2 (Zhang)

A

- Raw LFP
- Amplitude

SIA epochs

LIA epochs

Firing rate histogram

B

C

D

SIA index = SIA Z-score * FR_{onset}/FR_{base}
Figure 3 (Zhang)

A

Raster plot

Firing rate (Hz)

Time (sec)

Auditory stimulus (2sec)

B

Log$_{10}$(Firing rate)

Unit # (sorted)

Time (sec)

C

Neuron count

Aud index = FR$_{peak}$ / FR$_{base}$

D

Upper panels
Pro-arousal neurons

Lower panels
Other slow-firing neurons

Distractor Cue
Irrelevant Cue
Rewarded Cue

Log$_{10}$(Firing rate)
Figure 6 (Zhang)

A. LFP Phase-locking Z values

\[ t \text{ shift} = +40\text{ms} \]

B. Z value vs. Time shift (ms)

- HP_LFP
- MS_LFP

C. Neuron number vs. Time (ms)

- Pro-Arousal slow-firing
- Other slow-firing
- Fast-firing

- WK
- REM
- WK&REM
Figure 7 (Zhang)

A

Normalized cross-correlation

Proportion of significant cross-correlation (%)

Average cross-correlation

Time (sec)
Figure 8 (Zhang)

A  Pro-arousal  Other slow-firing  B  Pro-arousal  Other slow-firing  Pro-arousal  Other slow-firing

REM

Frequency (Hz)

300
200
100
20
10
0
0  5  0  5  0  5  0  5  0  5

WK

Frequency (Hz)

300
200
100
20
10
0
0  5  0  5  0  5  0  5  0  5

SW

Frequency (Hz)

300
200
100
20
10
0
0  5  0  5  0  5  0  5  0  5