Characterization of functionally distinct basal forebrain cholinergic cell types

PhD thesis

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Introduction

Basal forebrain cholinergic neurons (BFCNs) have been associated with a wide variety of cortical processes from synaptic plasticity, learning, and memory, to the modulation of sleep-wake cycle, brain states and oscillations. The timescale of these processes can vary from fast, millisecond-based to slow, second, minute or even hour-based neuronal firing changes. The cholinergic system is capable of fast, phasic reactions – for instance to a sudden sensory input – or slow, tonic activity changes, modulating brain states by altering acetylcholine concentration in the cortex. To control these markedly different processes, cholinergic cells are expected to exhibit a large set of firing patterns, in course of which the synchronous or asynchronous co-firing of individual units enables the system to produce adequate cholinergic output for every different modulatory scenario. Hypothetically speaking, this broad temporal modulatory output (from fast phasic to slow tonic actions) by the BFCNs can be achieved in two ways. By their firing abilities they can be either "generalists", meaning that individual cholinergic cells express all types of activity patterns, or they are "specialists", so that there are subgroups among them, each supporting a specific function. Earlier in vitro characterized two distinct types of firing patterns among BFCNs, the so-called early and the late firing neurons. The early firing neurons are more excitable, and show strong spike frequency adaptation; moreover, they are capable of reaching depolarization blockade. In contrast, the late firing neurons are less excitable with the ability to maintain this low frequency discharge rate for prolonged periods. Functionally, the early firing cells are better suited for fast, phasic changes causing a sudden acetylcholine release in the cortical target areas triggered by a sudden sensory input. This fast cholinergic mechanism is a key feature in the generation of cortical processes such as learning (especially reinforcement learning), attention, synaptic plasticity, and memory. Conversely, cortical acetylcholine release can be controlled by slow, tonic activity changes of the late firing neurons, regulating cortical processes such as arousal on a longer timescale. The distinct electrophysiological properties of the cholinergic neurons are in accordance with the diverse cholinergic modulatory functions, exhibiting phasic and tonic activity changes in the cortex. The in vitro data suggest the existence of distinct BFCN subgroups, but these have not yet been linked to in vivo functions. Whether "generalist" BFCNs are producing all the distinct functional modes or various "specialist" BFCN types can be attributed to segregated functions is still a debate.

We addressed this by conducting in vitro and in vivo measurements of BFCNs. BFCNs' intracellular properties were probed by precisely controlled in vitro experiments. These measurements were designed to characterize the electrical properties of the BFCNs by controlling their input parameters while testing if they are capable of exhibiting phasic and tonic firing patterns for a given input. Uncovering the correlation between BFCNs' firing and distinct behavioral processes, and if they synchronize their activity with each other, required in vivo behavioral measurements where multiple cholinergic single-unit activity can be registered simultaneously with cortical local field potential (LFP) recordings during controlled behavioral events.

BFCNs can respond to salient sensory inputs with short, 18 milliseconds (ms) latency, and high temporal precision. Therefore, we had to measure the stimulus presentations to the animal with the same accuracy, to precisely align the evoked action potentials (APs) to these events. To achieve this, we designed an in vivo data acquisition setup precise enough to operate at millisecond order temporal resolution during the delivery of cue and feedback stimuli, which allowed us to train the animals on specific learning tasks while measuring concurrent neuronal activity. Additionally, we registered the specific responses of the animals (such as licking for water) in parallel with the registration of their neuronal activity. This experimental setup enabled us to test both the electrophysiological and the functional heterogeneities among the cholinergic cells.

Objectives

The following specific questions was addressed in the thesis:

1. Determine if separate groups exist among the identified basal forebrain cholinergic cells based on their in vivo firing properties.

2. Address the synchronization among the BFCNs and with cortical oscillations.

3. Address the effect of BFCN activation on the outcomes of an auditory detection task. Therefore, we tested the influence of BFCNs coupling their firing with cortical activation on the performance of the animal during auditory conditioning. We correlated the distinct BFCN subgroups' firing to the timing and the outcome of a behavioral event.

Methods

Experimental methods: Adult (age >2months) ChAT-Cre (n=15, 14/15 male), ChAT-ChR2 (n=3, 3/3 male) and PV-Cre (n=4, 4/4 male) mice were used for behavioral recording experiments under the protocol approved by the Cold Spring Harbor Laboratory Institutional Animal Care and Use Committee in accordance with the National Institutes of Health regulations. ChAT-Cre mice (male, n=3, age >2months) were used for *in vivo* and ChAT-Cre mice (n=12, 7/12 males, P50-150) were used for *in vitro* recordings according to the regulations of the European Community's Council Directive of 24 November 1986 (86/609/EEC); experimental procedures were reviewed and approved by the Animal Welfare Committee of the Institute of Experimental Medicine, Budapest and the Committee for Scientific Ethics of Animal Research of the National Food Chain Safety Office. See also Nature Research Reporting Summary.

Surgical procedures, viral injection, microdrive construction and implantation, recording, optogenetic tagging and histology have been described previously. Mice were trained on one of two versions of an auditory head-fixed detection task. In the operant version, mice had to detect pure tones in a go/no-go paradigm as described in. In the Pavlovian version, mice responded to reward- and punishment-predicting pure tones with anticipatory licking. In this version, air-puff punishment was delivered in a fixed proportion of trials in each trial type, irrespective of the anticipatory lick response of mice.

Analysis of *in vivo* experiments. Spike sorting was carried out using MClust (A.D. Redish). Only neurons with isolation distance >20 and L-ratio (a cluster quality measure based on Mahalanobis distance) <0.15 were included. Optogenetic tagging was verified using the SALT. Putative cholinergic neurons were selected based on hierarchical cluster analysis of punishment response properties (response magnitude, PETH correlation with identified cholinergic neurons and PETH similarity scores with templates derived from groups of all unidentified cells and unidentified cells suppressed after punishment). These analyses have been described in detail previously. ACGs were calculated at 0.5ms resolution. ACG graphs were smoothed by a 5-point (2.5ms) moving average for plotting. When plotting all or average ACGs per group, individual ACGs were mean normalized and sorted using burst index (Burst-BFCNs) or refractory period (Reg-BFCNs). The burst index was calculated inspired by the algorithm introduced by the Buzsaki lab: the difference between the maximum ACG for lags of 0–10ms and the mean ACG for lags of 180–200ms was normalized by the larger of the two numbers, yielding an index between −1 and 1. The selectivity index for bursts and single spikes was calculated as the burst or single spike number in 20–50 ms relative to 100–250 ms post-event windows. It was not calculated for neurons that did not have bursts/single spikes in these windows due to an insufficient quantity of data. The theta index was calculated as the normalized difference between the mean ACG for a ±25-ms window around the peak between lags of 100 and 200ms (corresponding to a 5- to 10-Hz theta band) and the mean ACG for lags of 225–275 and 65–85ms. Normalization was performed similar to that for the burst index. The relative refractory period was defined as a low spiking probability after an AP had been fired, and was calculated by estimating the central gap in the ACG. To estimate the range of delays after an AP at which spiking happened with lower probability, we calculated the maximal bin count of the ACG smoothed by a 10-ms moving average, and

took the delay value at which the smoothed ACG first reached half of this value (width at half-height). We note that this definition captures low spike probability and not biophysical partial repolarization. As this algorithm allows APs in the 'refractory period', we used the term 'relative refractory period' (lower probability of firing). Nevertheless, this property captured the distinction between regular rhythmic and bursting neurons well. Cross-correlations (CCGs) were calculated at 1-ms resolution. Segments (±100ms) around reinforcement events were excluded to avoid trivial event-driven correlations; 0-ms lag (middle) values were excluded to avoid potential contamination from spike sorting artifacts. When plotting all or average CCGs, individual CCGs were Z-scored and smoothed by a 15-point moving average. Co-activation was considered significant if raw CCGs crossed the 95% confidence limits, calculated by the shift predictor method, for at least two consecutive bins. PETHs were averaged from binned spike rasters and smoothed by a moving average. For comparisons of bursts and single spikes, PETHs were divided by (1 + average baseline PETH). All PETHs were baseline subtracted for visual comparison. LFP recordings were carried out in the primary auditory cortex (A1) simultaneously with the tetrode recordings using platinum–iridium stereotrodes. LFP traces were Z-scored and averaged in windows centered on the APs of interest for STAs. Positive-deflecting STA traces were inverted before averaging for coherence because the depth of recording was not precisely controlled; therefore, we could not draw conclusions from absolute delta phases. Wavelet calculations were performed using the Morlet wavelet and STSs were calculated from the wavelet power and phase spectra. Individual frequencies were normalized by their averages to give equal weight to spectral components and visualized on a decibel scale. Note that this normalization method may introduce negative STS values.

In vitro recordings. Mice were decapitated under deep isoflurane anesthesia. The brain was removed and placed into an ice-cold cutting solution, which had been bubbled with 95% O_2–5% CO_2 (carbogen gas) for at least 30min before use. The cutting solution contained the following (in mM): 205 sucrose, 2.5 KCl, 26 $NaHCO_3$, 0.5 $CaCl_2$, 5 $MgCl_2$, 1.25 NaH_2PO_4, 10 glucose. Coronal slices of 300-µm thickness were cut using a Vibratome (Leica VT1000S). After acute slice preparation, slices were placed into an interface-type holding chamber for recovery. This chamber contained standard ACSF solution at 35 °C which gradually cooled down to room temperature. The ACSF solution

contained the following (in mM): 126 NaCl, 2.5 KCl, 26 $NaHCO_3$, 2 $CaCl_2$, 2 $MgCl_2$, 1.25 NaH_2PO_4, 10 glucose, saturated with 95% O_2–5% CO_2. Recordings were performed under visual guidance using differential interference contrast microscopy (Nikon FN-1) and a 40× water-dipping objective. Cholinergic neurons expressing ChR2-mCherry were visualized with the aid of a mercury arc lamp and detected with a CCD camera (Hamamatsu Photonics). Patch pipettes were pulled from borosilicate capillaries (with inner filament, thin-walled, outer diameter (OD) 1.5) with a PC-10 puller (Narishige). The composition of the intracellular pipette solution was as follows (in mM): 110 potassium gluconate, 4 NaCl, 20 4-(2-hydroxyethyl)- 1-piperazine-ethanesulfonic acid, 0.1 (ethylenebis(oxonitrilo))tetra-acetate, 10 phosphocreatine, 2 ATP, 0.3 GTP, 3mg ml^{-1} of biocytin adjusted to pH 7.3–7.35 using KOH (285–295mosmol l^{-1}). Recordings were performed with a Multiclamp 700B amplifier (Molecular Devices), low-pass filtered at 3 kHz, digitized at 10–20 kHz with NI USB-6353, X Series DAQ, and recorded with an in-house data acquisition and stimulus software (courtesy of Attila Gulyás, Institute of Experimental Medicine, Budapest, Hungary). For *in vitro* light illumination, we used a blue laser diode (447nm, Roithner LaserTechnik GmbH) attached to a single optic fiber (Thorlabs) positioned above the slice.

Analysis of *in vitro* experiments. All *in vitro* data were processed and analyzed off-line using self-developed programs written in Python v.2.7.0 and Delphi v.6.0 by A.I.G. and D.S. Spike delay was defined as the time between the start of the 1-s-long positive current injection step and the peak time of the first following AP. Burst frequency was calculated from the following three ISIs. The membrane potential was calculated as the average membrane potential of a 1-s-long period preceding the positive current injection step. ACGs for each cell were calculated on spikes evoked by step protocols and were smoothed by a 5-ms moving average. Step protocols form each cell were classified into three groups. Burst indices were calculated in a similar way to the *in vivo* recordings: the difference between the maximum ACG for lags of 0–15ms and the mean ACG for lags of 50–300ms was normalized by the larger of the two numbers, yielding an index between −1 and 1. The average burst index as a function of AP distance from bregma was calculated as a three-section moving average.

Statistics. No statistical methods were used to predetermine sample sizes, but our sample sizes are similar to those reported in previous publications. The present study did

not involve separate experimental groups; therefore, randomization and blinding across groups were not relevant. Behavioral trials were presented in randomized order. Data analysis was automated, irrespective of neuron identity. Putative single neurons with isolation distance >20 and L-ratio <0.15 were included in the *in vivo* analysis. These criteria were pre-established based on recommendations and standards of the field. In addition, the selectivity index could not be calculated for neurons that did not show any bursts or single spikes in the analyzed data window. If the number of recorded spikes exceeded 50,000, ACGs, CCGs, STAs and STS analyses were restricted to 50,000 spikes to avoid out-of-memory errors. We used nonparametric tests, therefore these neurons were pooled and resulted in a dataset of 78 BFCNs. Previous *in vitro* studies suggested that cholinergic neurons may exhibit heterogeneous firing patterns, however, this has not been tested *in vivo* and the potential diversity of BFCNs is unexplored in awake animals. We noticed that some cholinergic neurons were capable of firing bursts of action potentials *in vivo* with short, <10ms interspike intervals (ISIs), whereas others exhibited a markedly different pattern of regular rhythmic firing dominated by long ISIs. To quantify this, we defined relative refractory periods of basal forebrain cholinergic neurons for comparing central tendencies of two distributions, because normal distribution of the underlying data could not be determined unequivocally. For unpaired samples, the two-sided Mann–Whitney U-test was applied. For paired samples, we used the two-sided Wilcoxon's signed-rank test. Correlations were calculated using Pearson's correlation and tested using the one-sided F-test. Distributions over categorical variables were compared using the $\chi 2$ test for homogeneity. We tested the significance of optogenetic tagging using the SALT, which is a bootstrap test based on the Jensen– Shannon divergence of spike time distributions with or without stimulation.

RESULTS

Distinct firing patterns of cholinergic neurons *in vivo.* We performed extracellular tetrode recordings from the BF of awake mice. Cholinergic neurons were identified using an optogenetic tagging approach. Neurons responding with statistically significant short latency firing (stimulus-associated spike latency test (SALT): $P<0.01$) to blue laser light in transgenic mice expressing the photosensitive channelrhodopsin (ChAT-Cre infected by AAV-DIO-EF1a-ChETA, n = 15 or by AAV-DIO-EF1a-hChR2(H134R), n = 3; or

ChAT-ChR2, n = 3 mice) were considered to be optogenetically identified cholinergic neurons (n = 56). In addition, neurons that fell in the same cluster by hierarchical clustering of response properties were considered to be putative cholinergic neurons (n = 22). We detected no systematic differences between optogenetically identified and putative cholinergic neurons, therefore these neurons were pooled and resulted in a dataset of 78 BFCNs. Previous *in vitro* studies suggested that cholinergic neurons may exhibit heterogeneous firing patterns, however, this has not been tested *in vivo* and the potential diversity of BFCNs is unexplored in awake animals. We noticed that some cholinergic neurons were capable of firing bursts of action potentials *in vivo* with short, <10ms interspike intervals (ISIs), whereas others exhibited a markedly different pattern of regular rhythmic firing dominated by long ISIs. To quantify this, we defined relative refractory periods of basal forebrain cholinergic neurons based on their auto-correlograms, characterized by low probability of firing. The distribution of the relative refractory period duration covered a broad range (1–137 ms) and showed a bimodal distribution with two distinct, approximately log-normal modes. This was confirmed by a model selection approach based on Akaike and Bayesian information criteria. This demonstrated the existence of a separate, short-refractory, burst-firing and long-refractory, regular-firing group of cholinergic neurons. Therefore, we called these cholinergic neurons Burst-BFCNs and Reg-BFCNs, respectively. We further analyzed the burst-firing properties of Burst-BFCNs and found considerable heterogeneity based on their spike autocorrelations (ACGs). Many short-refractory neurons exhibited strongly bursting patterns with classic 'burst shoulders' in their auto-correlograms (Burst-BFCN-SBs, strongly bursting), whereas others showed irregular patterns of ISIs, resembling a Poisson process (Burst-BFCN-PLs, 'Poisson like'). Of note, the lack of a central peak in the autocorrelation did not preclude the occasional presence of bursts. These firing patterns were, on average, distinct; however, this separation was less evident than the bimodal relative refractory distribution, and a few neurons could have been categorized in either group. We note that the long-refractory neurons exhibited strong rhythmicity in the theta frequency band (5–10 Hz). The strength of rhythmic firing, quantified based on autocorrelation peaks in the theta band (theta index), correlated with the length of the relative refractory period (Pearson's correlation, P = 0.0007, one-tailed F-test). Next, we analyzed the firing patterns of a large dataset of untagged BF neurons. Burst firing has

been shown for GABAergic BF neurons before in agreement with this, we found that many (SB: n=559, PL: 692) noncholinergic cells were capable of burst firing. Surprisingly, however, only a small proportion of untagged BF neurons showed regular rhythmic firing with a long refractory period (n = 17). These neurons were similar to those that we had characterized as cholinergic (n = 12). This suggests that at least about 40% of regular rhythmic BF neurons are cholinergic, and may provide the means to identify this subgroup of putative cholinergic neurons based on firing rate and regular rhythmic activity pattern, when their response to air-puffs is not available.

In vitro recordings confirmed two types of cholinergic neurons. We wondered whether the different cholinergic firing patterns observed in our *in vivo* recordings reflect intrinsic properties produced by distinct cell types. Alternatively, distinct firing patterns may be determined by the current state of the network or variations in the input strength of individual cells. To answer this, we turned to *in vitro* preparations, where the membrane potential of the neuron and the strength of activation are precisely controlled and monitored. We performed whole-cell patch clamp recordings from n=60 cholinergic neurons from the BF in acute slices. Cholinergic neurons were identified by their red epifluorescence in n=12 mice injected with AAV2/5-EF1a-DIO-hChR2(H134R)-mCherry-WPRE-HGHpA.

We applied a somatic current injection protocol containing a 3-s-long, incremental 'prepolarization' step followed by a positive square pulse (1 s), to elicit spiking starting from different membrane potentials. We found two distinct behaviors on current injection using similar testing conditions. Cholinergic cells from the first group (red, n=29) displayed a short spike delay (8.05±0.74ms, median±s.e. of median) and bimodal ISI distribution with short ISIs corresponding to high-frequency 'burst' firing (maximum, 122.69±18.99Hz). The second group (green, n=31) displayed low maximal firing rate (13.81±2.32Hz, $P=1.54\times10^{-11}$, two-sided Mann–Whitney U-test), unimodal ISI distribution and a prominent spike delay (maximum spike delay, 153.05±55.59ms, 2.08×10^{-11} compared with the first group; two-sided Mann–Whitney U-test) which depended on the membrane potential before spiking. Importantly, depolarized late-firing cells responded to suprathreshold current injections with a short spike delay as opposed to the hyperpolarized state where late firing was prominent.

These distinct early responding/burst-firing or late responding/nonbursting modes were also reliably elicited by optogenetic depolarization. Spontaneous action potentials revealed shorter spikes and large-amplitude, slowly decaying afterhyperpolarization in late-firing compared with early-firing (bursting) cells. To compare *in vivo* and *in vitro* firing patterns, we calculated ACGs and burst indices (early-firing, 0.64±0.08; late-firing,−1.0±0, 2.11×10^{-12}, two-sided Mann–Whitney U-test) from spike trains during the current injection protocol. Early and late-firing neurons *in vitro* matched Burst-BFCNs and Reg-BFCNs *in vivo,* suggesting that these groups are the same. Next, we tested whether the different *in vivo* firing modes of bursting cholinergic neurons (Burst-BFCN-SBs versus Burst-BFCN-Pls) could be explained by variations in the membrane potential and input strength. To investigate this possibility, we applied somatic current injection protocols designed to test input and state dependency of the degree of bursting. Indeed, we found that the same Burst-BFCNs were capable of producing both strongly bursting and Poisson-like firing patterns. This property depended on both the membrane potential of the neuron and the strength of the activation, with Poisson-like firing occurring more frequently in more depolarized states and in response to stronger depolarizing inputs. In summary, we identified two types of BFCNs. Reg-BFCNs showed regular theta-rhythmic firing *in vivo* and late, regular responses to current injections *in vitro*; Burst-BFCNs exhibited burst firing both *in vivo* and *in vitro*, where the strength of bursting was determined by the level of excitation.

Cholinergic bursts transmit phasic information about reinforcers. Cholinergic neurons act at different timescales regulating different aspects of cognition, from slow sleep–wake and arousal processes to fast subsecond or even millisecond timescales of reinforcement learning and plasticity. Based on *in vitro* studies it was hypothesized that bursting specifically represents fast ‘phasic’ information transfer, however, this has not been tested. Therefore, we analyzed the activity of basal forebrain cholinergic neurons after reward and punishment in mice performing auditory conditioning. We defined a burst as a series of action potentials starting with an ISI <10ms and subsequent ISI durations of <15ms to allow for typical ISI accommodation patterns. As expected, Burst-BFCNs, categorized based on auto-correlograms, showed a high percentage of burst firing: 28% for Burst-BFCN-SBs and 20% for Burst-BFCN-PLs, whereas little burst activity was detected in Reg-BFCNs (3%). We have shown previously that the strongest

response of cholinergic neurons occurred after air-puff punishments: BFCNs responded phasically with short latency (18±1.9ms, median±s.e. of median), low jitter (5.7±0.1ms) and high reliability (81.7±2.6%). In the present study, we compared bursting and regular rhythmic cholinergic neurons, and found that both types showed strong response to air-puff punishment. Contrary to previous hypotheses, Reg-BFCNs were also capable of surprisingly fast and precise phasic firing, emitting a precisely timed single action potential, typically followed by a pause and then a reset of their intrinsic theta oscillation. This clearly distinguished them from tonically active striatal interneurons, which did not show such responses.

Burst-BFCNs are capable of emitting both bursts of action potentials and single spikes. Therefore, we wondered whether bursts and single spikes represent salient events such as air-puffs differently, in which case this should be reflected in a difference in peri-event time histograms (PETHs) of bursts versus single APs aligned to punishment events. We found that bursts of Burst-BFCNs significantly concentrated after punishment compared with single spikes in most neurons ($P=1.23\times10^{-6}$, two-sided Wilcoxon's signed-rank test). We observed similar concentration of bursts after reward, but not cue stimuli or trial start signals, suggesting that bursts represent external events differently compared with single spikes. *In vitro* studies also predicted that tonically active neurons would be more important in controlling slow tonic changes in acetylcholine levels, which could potentially be reflected in higher baseline firing rates of Reg-BFCNs. However, we found that baseline firing rates were largely similar across cholinergic cell types and firing patterns (median±s.e.: Burst-BFCN-SBs, 4.55±1.26; Burst-BFCN-PLs, 5.74±1.39; Reg-BFCNs, 3.96±1.0), with slightly faster firing in Burst-BFCN-PLs, consistent with the more depolarized membrane potentials and stronger excitatory inputs suggested by our *in vitro* recordings (Burst-BFCN-SBs versus Burst-BFCN-PLs, P=0.11; Burst-BFCN-SBs versus Reg-BFCNs, P=0.41; Burst-BFCN-PLs versus Reg-BFCNs; P=0.0236; two-sided Mann–Whitney U-test). Bursting cholinergic neurons show synchronous activity. Bursts of cholinergic neurons were found to precisely align to reinforcement, generating a strong synchronous activation of the cholinergic system after reward and punishment. Is synchronous firing specific to these unique behaviorally relevant events or do they occur at other times as well? Synchronous versus asynchronous activation of subcortical inputs has a fundamentally different impact on cortical computations. However, although there

is a lot known about synchrony in cortical circuits both within and across cell types, there is little information on synchronous firing in subcortical nuclei. Specifically, no recordings of multiple identified cholinergic neurons have been performed. In some cases, we recorded 2 (n=15) or 3 (n=3) cholinergic neurons simultaneously, resulting in 24 pairs of concurrent cholinergic recordings. By calculating pairwise cross-correlations, we found that Burst-BFCNs, especially Burst-BFCN-SBs, showed strong zero-phase synchrony with each other (6/6 pairs of two Burst-BFCN-SBs and 5/11 pairs containing Burst-BFCN-Sbs and -PLs showed significant co-activation, $P<0.05$). Reg-BFCNs showed little synchrony with other BFCNs (2/7 pairs that contained at least one Reg-BFCN were significantly co-activated, $P<0.05$, bootstrap test).

Co-activation of Burst-BFCNs typically spanned ±25ms (27.22±5.37, mean±s.e.m.; maximum 42ms) and was not restricted to the bursts themselves, because single action potentials of bursting neurons showed similar synchrony; thus Burst-BFCNs may share a synchronizing input that differentiates them from other BFCNs, possibly contributing to the bursting phenotype itself.

Cholinergic bursts are coupled to cortical activity. Cholinergic neurons send dense innervation to the cortex, including projections from the nucleus basalis (NB) to auditory cortices. These inputs can potently activate cortical circuits, leading to desynchronization and gamma oscillations which we confirmed by optogenetic stimulation of NB cholinergic neurons that elicited broad-band activity in the auditory cortical LFPs. We reasoned that bursts of cholinergic firing might lead to stronger cortical activation, whereas synchronous activation of ensembles of cholinergic neurons may further increase this effect, providing a finely graded control over cortical activation and thus arousal by the ascending cholinergic system. At the same time, the BF receives cortical feedback that may be capable of entraining cholinergic neurons, thus establishing an ongoing synchrony between cortical and BF activity, a hypothesis largely under-explored. To test these possibilities, we calculated spike-triggered LFP averages and spike-triggered spectrogram averages of auditory cortical LFPs aligned to the action potentials of BFCNs recorded during auditory operant conditioning. We used spike-triggered averages (STAs) to identify synchronization between BFCN spiking and cortical oscillations, because LFP changes not phase locked with BFCN spikes cancel out. Individual STAs aligned to cholinergic spikes showed prominent oscillations in the theta band (4–12Hz), suggesting

that NB cholinergic activity can synchronize to cortical theta oscillations. In addition, we often observed strong deflections in cortical LFPs after cholinergic spikes (peak latency, 36.0±13.0ms; median±s.e. of median), which may be a signature of cortical activation by cholinergic input.

We confirmed that artificial synchrony of BFCNs imposed by optogenetic or electrical stimulation induced cortical desynchronization, as shown previously. As we have found that synchronous activation of BFCNs also occurred in a physiological setting, this raises the question of whether such synchrony indeed leads to stronger cortical impact. To test this, we focused our analysis on the synchronous firing of cholinergic pairs. We found that synchronous events defined by two Burst-BFCNs firing within 10ms was associated with strong cortical activation compared with asynchronous firing, confirming our prediction that NB signatures of enhanced cholinergic release represent a stronger impact on cortical population activity. We observed that bursting cholinergic neurons often showed synchronization to cortical theta-band oscillations. The presence of high values in the theta band in the average spectral phase (phase domain of STSs) confirmed this, because it reflects phase locking to LFP oscillations. We reasoned that differential activation of cholinergic cell types by their inputs might underlie differences in synchronizing with cortical oscillations. It is known that frontal cortical projections to the BF synapse on GABAergic neurons, likely providing indirect hyperpolarizing input to cholinergic neurons. To model the impact of this circuit on BFCNs, we tested whether Burst-BFCNs and Reg-BFCNs show differential responses to hyperpolarizing current injections *in vitro*. We found that Burst-BFCNs recovered their spikes with shorter and less variable latency (n=4, 172.3±9.95ms, median±s.e. of median than Reg-BFCNs (n=6, 561.25±23.77ms; $6.47\times10-44$, two-sided Mann–Whitney U-test). This supports the hypothesis that cortically driven indirect inhibition of BFCNs may contribute to their differential coupling to cortical activity.

Synchrony of BFCN spiking with cortical activity predicts behavior during auditory detection. We have demonstrated that bursting and regular rhythmic cholinergic neurons are differentially coupled with the auditory cortex. However, the functional significance of this connection remains unclear. Therefore, we tested whether synchrony between BFCNs and the auditory cortex predicted behavioral performance during auditory conditioning. Specifically, we restricted our analysis to 1-s-long windows

around auditory cue presentation during the operant auditory detection task. We found that Burst-BFCNs, especially Burst-BFCN-SBs, showed larger STA deflections during hit and false-alarm trials compared with miss and correct-rejection trials.

Therefore, synchronization of Burst-BFCNs with cortical networks predicts mouse responses but not their accuracy, because correct and incorrect responses showed similar STAs. In contrast, we found that large STA deflections for Reg-BFCNs specifically predicted hits; thus, synchronization of Reg-BFCNs and the auditory cortex predicted performance. We did not find similar predictive activity in a 1-s window before the cues, suggesting that predictive synchronization of the BF and auditory cortex was evoked by the cue tones. In summary, we found a behavioral dissociation between the two cholinergic cell types; while cortical coupling of Burst-BFCNs preceded all responses of the animals regardless of performance, Reg-BFCNs specifically predicted correct responses.

The horizontal diagonal band contains few regular cholinergic neurons. We wondered whether the uncovered diversity of cell types is uniform across the basal forebrain; alternatively, differences in the distribution of bursting and regular rhythmic cholinergic neurons may suggest that dedicated cortical areas are differentially regulated by BF cholinergic afferents. The cholinergic neurons we recorded were distributed in the NB and in the more anterior horizontal limb of the diagonal band of Broca (HDB), spanning almost 2mm rostrocaudal distance. This allowed us to investigate whether BFCN types are differentially distributed along the anteroposterior axis of the BF. In our *in vivo* recordings, 27% (n=12/45) of the NB neurons belonged to the regular rhythmic type, whereas this was only 9% (n=3/33) for the HDB.

When we recorded NB neurons *in vitro*, 66% (n=27/41) were Reg-BFCNs, whereas only 22% (2/9) Reg-BFCNs were found in the HDB. The higher proportion of Burst-BFCNs in our *in vivo* recordings could be due to better cluster separation because of their somewhat higher firing rates and distinct spike shape. Nevertheless, we found that the NB contained three times more regular rhythmic cholinergic neurons both *in vivo* and *in vitro* compared with the HDB, which mostly contained the bursting type ($P=0.0007$, $\chi2=11.37$, $\chi2$ test). In line with these, the burst index and relative refractory period of cholinergic neurons changed systematically along the anteroposterior axis of the BF,

suggesting that different brain areas may receive different combinations of cholinergic inputs. Turning to untagged HDB neurons that we recorded *in vivo*, we found that only 12 of 560 HDB neurons were characterized as regular firing, which confirms both the lack of Reg-BFCNs in the HDB and the connection between regular rhythmic phenotype and cholinergic identity.

Conclusions

The main conclusions of my doctoral dissertation are the following:

(1) The cholinergic basal forebrain contains two distinct functional cell types characterized by either burst-firing or rhythmic, non-bursting firing patterns.
(2) Reg-BFCNs constitute about half (one-third to two-thirds) of BFCNs in the NB, whereas the more anterior HDB cholinergic neurons were mostly (80–90%) of the Burst-BFCN type.
(3) Regular firing cholinergic cells are incapable of firing bursts even in *in vitro* current injection experiments.
(4) Burst-BFCNs showed strong synchrony with each other and cortical oscillations, suggesting that they may have a strong impact on cortical processing.
(5) Synchrony between Burst-BFCNs and the auditory cortex at stimulus presentation predicted response timing.
(6) Coupling between Reg-BFCNs and the auditory cortex was strongest before mice made successful hits, thus predicting behavioral performance.

The bibliography of the candidate's publications

Publications related to the Ph.D. dissertation

1. **Laszlovszky, T.**, Schlingloff, D., Hegedüs, P., Freund, T. F., Gulyás, A., Kepecs, A., & Hangya, B. (2020). Distinct synchronization, cortical coupling and behavioral function of two basal forebrain cholinergic neuron types. *Nature Neuroscience*, *23*(8), 992–1003.
IF: 24,884

2. Solari, N.[†], Sviatkó, K. [†], **Laszlovszky, T.** [†], Hegedüs, P. [†], & Hangya, B. (2018). Open source tools for temporally controlled rodent behavior suitable for electrophysiology and optogenetic manipulations. *Frontiers in Systems Neuroscience, 12*(May), 1–14.

[†] Equal contribution

IF: 3,928

Other publications

Domonkos, A., Nikitidou Ledri, L., **Laszlovszky, T**., Cserép, C., Borhegyi, Z., Papp, E., Nyiri, G., Freund, T. F., & Varga, V. (2016). Divergent *in vivo* activity of non-serotonergic and serotonergic VGluT3–neurones in the median raphe region. *Journal of Physiology*, *594*(13), 3775–3790.

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