Activation of the Cholinergic System Endows Compositional Properties to Striatal Cell Assemblies

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Carillo-Reid L, Tecuapetla F, Ibáñez-Sandoval O, Hernández-Cruz A, Galarraga E, Bargas J. Activation of the cholinergic system endows compositional properties to striatal cell assemblies. J Neurophysiol 101: 737–749, 2009. First published November 19, 2008; doi:10.1152/jn.90975.2008. Striatal cell assemblies are thought to encode network states related to associative learning, procedural memory, and the sequential organization of behavior. Cholinergic neurotransmission modulates memory processes in the striatum and other brain structures. This work asks if the activity of striatal microcircuits observed in living nervous tissue, with attributes similar to cell assemblies, exhibit some of the properties proposed to be necessary to compose memory traces. Accordingly, we used whole cell and calcium-imaging techniques to investigate the cholinergic modulation of striatal neuron pools that have been reported to exhibit several properties expected from cell assemblies such as synchronous states of activity and the alternation of this activity among different neuron pools. We analyzed the cholinergic modulation of the activity of neuron pools with multidimensional reduction techniques and vectorization of network dynamics. It was found that the activation of the cholinergic system endows striatal cell assemblies with properties that have been posited for recurrent neural artificial networks with memory storage capabilities. Graph theory techniques applied to striatal network states revealed sequences of vectors with a recursive dynamics similar to closed reverberating cycles. The cycles exhibited a modular architecture and a hierarchical organization. It is then concluded that, under certain conditions, the cholinergic system enables the striatal microcircuit with the ability to compose complex sequences of activity. Neuronal recurrent networks with the characteristics encountered in the present experiments are proposed to allow repeated sequences of activity to become memories and repeated memories to compose learned motor procedures.

INTRODUCTION

The basal ganglia participate in the acquisition, storage, and execution of motor skills that commonly involve sequences of movements (Aosaki et al. 1994; Cools 1980; DeLong 1981). The main stage of the basal ganglia is the striatum, which displays a dense staining for cholinergic markers (Holt et al. 1997). At the cellular level, cholinergic interneurons enhance the excitability of neostriatal projection neurons through the activation of muscarinic receptors that regulate several voltage-dependent ion currents (e.g., Calabresi et al. 1998; Figueroa et al. 2002; Galarraga et al. 1999; Perez-Burgos et al. 2008; Shen et al. 2005). At the same time, presynaptic muscarinic receptors modulate the strength of the connections that these neurons exert on their targets (Perez-Rosello et al. 2005). In addition, acetylcholine enhances the activity of striatal interneurons (Koos and Tepper 2002) that play a prominent role in cell assembly orchestration (Berke et al. 2004; Carrillo-Reid et al. 2008). Therefore it is difficult to visualize a priori how all these cholinergic actions combine at the microcircuit level. Accordingly, this study is aimed at describing cholinergic actions in the striatal microcircuit as a first step toward a top-down approach to link the activity in small networks with cellular physiology.

The cholinergic system has long been associated with cognitive functions and memory processes in the striatum (Calabresi et al. 1998; Diaz del Guante et al. 1991) and other brain regions (Hasselmo and Giocomo 2006). Cholinergic modulation changes the ability of animals to switch among different behaviors (Cools 1980), and the activity of the striatal cholinergic system enables temporal windows for procedural learning to take place (Morris et al. 2004).

Nevertheless, possible neuronal microcircuits that may function as correlates for cognitive abilities and their cholinergic modulation are hard to demonstrate in living brain tissue. For example, to manage memory storage during cognitive processes several types of neuronal circuitries have been posited, such as Hebbian cell assemblies, synfire chains or closed reverberating circuits whose neurons should be capable of synchronization and alternation of their correlated activity among diverse neuronal pools, thus conforming functional cycles of recurrent activity (Abeles 2003; Hammer 2003; Hebb 1949; Lorente de No 1938). Neuronal microcircuits with the potential to behave in this way have rarely been observed directly in the mammalian brain tissue (e.g., Carrillo-Reid et al. 2008; Ikegaya et al. 2004; Reyes 2003) or even in artificial networks (Hammer 2003). Therefore their functioning and capabilities have not been fully described. As an approximation, however, multi-unitary electrophysiological recordings, associated with field recordings, have inferred the workings of recursive cell assemblies based on their correlated firing (Abeles 2003; Beiser et al. 1997; Berke et al. 2004; Dragoi and Buzsaki 2005).

In the present study, we show, in the neostriatal brain slice preparation, neuronal pools with several attributes expected from Hebbian-like cell assemblies capable of memorization (Abeles 2003). First, we observed these neuronal pools directly by combining whole cell electrophysiological recordings and dynamic multicellular calcium imaging. Second, we used multidimensional reduction of network dynamics to rigorously...
compare the activity of neuronal pools over time. Third, we used hard and fuzzy clustering algorithms, taking the Dunn’s index as a validity function, to depict the different states of the network in a plane (Bezdek et al. 1997; Carrillo-Reid et al. 2008; Sasaki et al. 2007). Finally, we used graph theory, applied to depicted network states, to show that there are closed reverberating cycles that exhibit a modular structure and a hierarchical organization as assessed by graph-based hierarchival conceptual clustering (Jonyer et al. 2001). Interestingly, the analysis demonstrate that striatal microcircuits present the cardinal property of compositionality, which refers to the ability of a given system to build complex hierarchical representations, in terms of simpler parts and their relationships (Bienenstock and Geman 1995; Hammer 2003).

METHODS

Slice preparation

Transverse corticostriatal slices (300 μm thickness), were obtained from PD14-18 Wistar rats as described in previous work from our laboratory (Carrillo-Reid et al. 2008; Vergara et al. 2003). All procedures conformed to the guidelines of the Universidad Nacional Autónoma de México’s Animals Scientific Procedures Committee. Slices were obtained with ice-cold saline (4°C) containing (in mM): 123 NaCl, 3.5 KCl, 1 MgCl2, 1 CaCl2, 26 NaHCO3, and 11 glucose (25°C; saturated with 95% O2–5% CO2, pH = 7.4; 298 mosM/l). Slices were then transferred to saline at room temperature (21–25°C) where they remained for ≥1 h before recording.

Calcium imaging

Slices were loaded for calcium imaging by incubation at room temperature in the dark for 20 min with 10 μM fluo-4 AM (Tef Labs, Austin, TX) in 0.1% dimethylsulphoxide (35°C), equilibrated with 95% O2–5% CO2. Slices were bathed with control saline (see preceding text) in a perfusion chamber placed on the stage of an upright microscope equipped with a ×20, 0.95 NA water-immersion objective (Olympus BX51WI; Olympus). Illumination pulses at 488 nm (50-100-ms exposure) were delivered to the preparation with a Lambda LS illuminator (Sutter instruments, Novato, CA), connected to the microscope via fiber optics. Experiments were performed at room temperature.

Images were acquired with a cooled digital camera (CoolSNAP ES2, Photometrics, Roper Scientific, Tucson, AZ) at 250–500 ms using RS Image (Photometrics; Roper Scientific). The imaged field was 800 × 600 μm in size. Short movies (~250 s) were taken at time intervals of 5–20 min during 1 h.

The number of fluo-4-loaded neurons in the field was determined at the end of the experiment with a brief exposure (5 s) to saline containing 50 mM KCl. This maneuver disclosed all fluo-4-labeled neurons (either active or silent during the experiment). Cells active during the experiment were identified, and the ratio of active versus silent cells was obtained. Spontaneous or evoked calcium transients, together with voltage responses were recorded electrophysiologically in some cells, both in control saline and during the application of different drugs (see RESULTS). This allowed us to study changes of network activity under different pharmacological conditions.

Drugs

Stock solutions were prepared before each experiment and added to the perfusion solution in the final concentration indicated. N-methyl-D-aspartate (NMDA), muscarine chloride, eserine and atropine sulfate were obtained from Sigma (St. Louis, MO).

Electrophysiology

An Axoclamp 2B amplifier (Axon Instruments, Foster City, CA) was used to perform whole cell current- and voltage-clamp recordings. Signals were filtered at 1–3 kHz and digitized at 3–9 kHz with an AT-MIO-16E4 board (National Instruments, Austin, TX) in a PC computer. Data acquisition used software designed in the LabView environment. Patch pipettes (3–6 MΩ) were filled with (in mM): 115 KH2PO4, 2 MgCl2, 10 HEPES, 0.5 EGTA, 0.2 Na2ATP, and 0.2 Na2GTP. In some experiments, biocytin 0.5%, and fluo-4 salt (10–20 μM) were added to the recording pipettes.

Image analysis

Image processing was done as previously described (Carrillo-Reid et al. 2008) using Image J (v.1.36, National Institutes of Health) and custom-made programs written in IDL (Cossart et al. 2003; Mao et al. 2001) and MATLAB (The Math-Works, Natick, MA). Briefly, active neurons were semi-automatically identified, and their mean fluorescence was measured as a function of time. Calcium-dependent fluorescence signals were computed as (F1 − F0)/F0, where F1: fluorescence intensity at any frame, and F0: resting fluorescence, i.e., average fluorescence of the first 4 frames of the movie. Calcium signals elicited by action potentials were detected based on a threshold value given by their first time derivative (2.5 times the SD of the noise value). Recordings were inspected manually to remove artifacts (Carrillo-Reid et al. 2008; Ikegaya et al. 2004; Sasaki et al. 2007).

Statistical methods

IDENTIFYING NETWORK STATES. The method employed to detect striatal network states has been published (Carrillo-Reid et al. 2008). Briefly, to determine if neuronal activity as observed by calcium transients and recorded from different cells simultaneously was correlated, the numbers of simultaneous activations per trial were detected. To determine the P value of simultaneous calcium transients occurring by chance, the distribution under the null hypothesis of independent transients using Monte Carlo simulations with 1,000 replications were computed (Mao et al. 2001).

The degree of firing correlation between active cells was calculated with the Jaccard correlation coefficient. Correlation maps were constructed where line thickness was proportional to the correlation coefficient. We also constructed pseudocolored cross-correlation maps to directly inspect all correlated cell pairs. To identify peaks of synchronous activity (i.e., including more cells than those expected by chance), Monte Carlo simulations were also used to estimate the significance of concurrent firing. The chosen threshold corresponded to a significance level of P < 0.01. Peaks of synchronous activity that remained significant during the experiment were selected for further analysis.

To identify the neurons underlying each network state and their dynamics, D × N matrices were constructed, where D represents the number of active neurons in a set of experiments and N denotes the firing of cells during 250-ms to 1-s time bins (NMDA-induced up-states last between 0.5 and 5 s) (Carrillo-Reid et al. 2008). In this manner, peaks of synchronous activity were vectorized so that bursting over time of all active cells with correlated firing during the time bin represent the elements of each vector, corresponding to a network state. Neuron pools represent the cells from which specific network states arise. We previously showed that first time derivatives of the calcium transients correspond to the duration of electrophysiologically recorded bursts or up-states in single neurons (Carrillo-Reid et al. 2008). Therefore the set and sequential activation of these vectors can be used to describe network activity as a function of time (Brown et al. 2005; Carrillo-Reid et al. 2008; Sasaki et al. 2007).
To identify if a given vector is formed by similar or different subsets of firing neurons, we measured a similarity index between all network vectors appearing during an experiment by computing the inner product (similarity index) of all possible vector pairs (Carrillo-Reid et al. 2008; Sasaki et al. 2007; Schreiber et al. 2003). Then we plotted the similarity indexes as a pseudocolored matrix in which functional states sustained by significantly correlated or synchronized neural vectors appear as cluster-like structures (Carrillo-Reid et al. 2008; Sasaki et al. 2007).

To follow network dynamics within the neuronal microcircuits, we reduced the dimensionality of the neural vectors with locally linear embedding (LLE), a technique for nonlinear dimensionality reduction (Brown et al. 2005; Roweis and Saul 2000; Stopfer et al. 2003). It was previously found that trajectories of high dimensional data are not well described with simple linear dimensionality reduction methods (Brown et al. 2005; Carrillo-Reid et al. 2008). In LLE, the vectors representing network states were projected into a vectorial space made up by the first two dimensions with data points representing vectors at a given time. A cluster of data points represent vectors with similar dynamics. Arrows joining clusters of data points indicate the trajectories followed by the synchronous activity involving different network states. To choose the optimal number of clusters appearing in an experiment, we used hard and fuzzy clustering algorithms taking the Dunn’s index as a validity function (Bezdek et al. 1997; Carrillo-Reid et al. 2008; Sasaki et al. 2007). To determine the number and identity of the neurons in each neuron pool belonging to each network state, hierarchical cluster analysis was computed using Euclidean distances and the nearest neighbor single linkage method (Systat, Richmond, CA).

IDENTIFYING CLOSED CYCLES OF ACTIVITY BETWEEN NETWORK STATES. We asked whether activity trajectories of neural vectors include reverberating loops characteristic of Hebbian-like cell assemblies and special classes of synfire chains (Abeles 2003; Harris 2005; Hebb 1949). Thus to visualize the trajectories of the synchronized activity between network states, we constructed isomorphic directed graphs (or digraphs) of network state transitions. A digraph is a graph formed by vertices and directed edges (arrows pointing to 1 endpoint) (Diestel 2005; Gerber 1990). Peaks of synchronous activity, belonging to specific network states, denote the vertices of the digraphs. Arrows denote the trajectories from one state to the other. Closed cycles were identified when they exhibit the properties of Hamiltonian or Eulerian cycles. A Hamiltonian cycle of a digraph (directed graph) G is a closed walk that contains every vertex of G exactly once. An Euler tour is a closed walk traversing every edge exactly once (Diestel 2005). As previously suggested (Grillner 2006; Hebb 1949), a closed cycle in a network is truly reverberating and capable of self-sustained activity in the absence of external stimuli when the traveling activity begins and ends at the same point (Hebbian closed cycle or phase sequence). Thus to analyze these cycles in the striatal microcircuit better, we constructed sentences that reflected network dynamics over time, such as “ababic,” which represents the state transitions from states a to b, b to c, c to a, a to b, and so on, where each letter represents a given network state (vertex of the digraph). Once a sentence was identified for a given experiment, we searched for the existence of closed cycles that form words. For example “abaca” is a word representing a closed cycle within the cited sentence in the preceding text. For each closed cycle found in the sentence, we obtained the adjacency matrix of the given digraph (Diestel 2005).

The adjacency matrix of a Hamiltonian digraph has the following property: \( H^T = I \), where \( n \) is the number of vertices of the digraph and \( I \) is the identity matrix. Hamiltonian cycles also have the properties of cyclic groups. The adjacency matrix of an Eulerian digraph \( E \) has the following property: \( \sum_{i=1}^{n} a_{ii} = \sum_{j=1}^{n} a_{jj} \), that is, the sum of the elements of the row \( i \) is equal to the sum of the elements of the column \( i \).

We used graph-based hierarchical conceptual clustering (Jonyer et al. 2001) to search for smaller units or semantic atoms (Abeles 2003; Bienenstock and Geman 1995; Hebb 1949) that could compose the complete representation of the striatal cycles (Bienenstock and Geman 1995).

Finally, we looked for cycles that could be performing low level or parallel processing. That is, cycles appearing in the activity of individual cells before they emerge as peaks of statistically significant synchronous activity (within a neural vector). The identification of such matching sequences in some cells revealed the same cycles found with the peaks of synchronous activity. Thus the same words of the sentences formed by the transitions between network states could be observed as running in low level (Lee and Wilson 2002), a property known as priming (Abeles 2003). Priming refers to the activation of individual cells forming specific sequences before enough neurons in a pool get synchronized to generate a significant peak of synchrony (neural vector). To find priming sequences in individual cells, we examined the onsets of their calcium transients. Thus priming sequences are the same sequences found in the cell assembly among neural vectors but running in low level.

RESULTS

Optical and electrophysiological recordings of striatal neurons

Figure 1A illustrates loaded cells within an observational field. Automatic contour detection allowed us to reconstruct a spatial map of the neurons (Fig. 1B; \( \rightarrow \), 1 cell targeted for electrophysiological recordings). Most loaded cells were medium spiny projection neurons (see Carrillo-Reid et al. 2008) (Fig. 1C). We previously showed that the firing of bursts comprising two or more action potentials produces discernible Ca\(^{2+}\) transients in projection striatal neurons, thus making it possible to monitor electrical activity of many cells by dynamic calcium imaging (Fig. 1D). Simultaneous electrophysiological and calcium-imaging recordings confirmed that the first derivative of the calcium signals can be used to reconstruct the time course of spontaneous bursts. Spontaneous Ca\(^{2+}\) transients are then transformed into raster plots (Carrillo-Reid et al. 2008) that can be used to follow simultaneously the activity of dozens of neurons with single-cell resolution. Under control conditions, medium spiny neurons are usually silent. Nevertheless, and as it has been exhaustively shown in several circuits of the nervous system (e.g., Grillner 2006; Guertin and Hounsgaard 1998; Hsiao et al. 1998; Ibañez-Sandoval et al. 2007; Tseng and O’Donnell 2005; Zhu et al. 2004), the tonic excitatory drive conveyed by NMDA application induces recurrent patterned bursting in striatal neurons (Carrillo-Reid et al. 2008; Vergara et al. 2003) similar to that found in vivo (Herrling et al. 1983) and correlated with movement execution (Grillner 2006; Ososwksa and Wolfarth 1995; Vautrelle et al. 2008), demonstrating that patterns of activity (motor behaviors) occurring in vivo could arise from the striatal processing of a tonic excitatory drive such as NMDA.

Given that cholinergic modulation in neostriatal neurons increases postsynaptic excitability, while at the same time decreases the strength of their GABAergic collateral synaptic connections (Figueroa et al. 2002; Galarraga et al. 1999; Perez-Rosello et al. 2005; Shen et al. 2005), we hypothesized that cholinergic modulation should increase the ability of cell assemblies to alternate their synchronized activity between neuronal pools within the striatum (Abeles 2003).
Cholinergic modulation of correlated firing in the striatal microcircuit

NMDA induced network dynamics in the striatum, that is, neuron pools with synchronized firing alternate their activity with other pools, to produce cycles of activity formed by network states evolving over time (Carrillo-Reid et al. 2008). We investigated the effects of increasing the cholinergic tone on these dynamics. Accordingly, we first studied the cholinergic modulation of firing patterns at the single-cell level with voltage recordings and, simultaneously, followed the activity of dozens of neurons with multicellular calcium imaging.

Figure 2 shows that after bath addition of muscarine (5 μM) there was a change in the firing pattern induced by NMDA (Fig. 2A). The frequency and level of membrane potential depolarization of spontaneous voltage transitions increased (n = 26 cells). The ionic basis that underlie this firing pattern deserves further analysis and perhaps includes the blockage of KCNQ-channels (Shen et al. 2005), the activation of cationic currents (Figuerova et al. 2002), and the blockage of the spike afterhyperpolarization (Perez-Burgos et al. 2008; Perez-Rosello et al. 2005). In this work, we show that the change in firing pattern is associated with an increase in the negative slope conductance region (NSCR) and a reduction in inward rectification (Figueroa et al. 2002; Galarraga et al. 1999) as is evident in the current-voltage relationship (Carrillo-Reid et al. 2008; Vergara et al. 2003) (Fig. 2B). The NSCR endows neurons with nonlinear capabilities (Izhikevich 2007). Increased spontaneous voltage transitions and calcium transients could be abolished by addition of the NMDA receptor antagonist AP5 (50 μM; data not shown), suggesting that the genera-
tion of the NSCR in striatal neurons is determinant for this activity (Carrillo-Reid et al. 2008; Vergara et al. 2003). Moreover, the muscarinic-dependent increase of the NSCR is only seen in the presence of NMDA (data not shown), the activation of muscarinic receptors in medium spiny neurons at rest (without activity induced by NMDA) only reduces the amount of inward rectification (Figueroa et al. 2002; Galarraga et al. 1999).

To study the actions of muscarine at the network level, we used multicellular calcium imaging to obtain brief movies (180...
s) under different conditions, and by means of raster plot analysis followed the activity of multiple cells simultaneously (Fig. 2C, top: each row in the raster plot represents an active neuron). The activation of muscarinic receptors increased considerably both the correlated firing among neurons (Fig. 2E) and the number of peaks of spontaneous synchronous activity (Carrillo-Reid et al. 2008; Cossart et al. 2003) (Fig. 2C bottom; n = 20 slices; 2.8 peak/epoch in NMDA vs. 12.2 peak/epoch in muscarine; P < 0.001). The timing of peaks of synchronous activity correlated with the timing of voltage-state transitions as measured electrophysiologically (Carrillo-Reid et al. 2008).

Active neurons are marked with filled circles in the maps of Fig. 2D. Blue labels indicate cells that were active before and during muscarine exposure. Red-filled circles indicate cells that were active during the peaks of synchrony. Lines connect pairs of neurons that had nonrandom correlated activity (P < 0.01: the thickness of these lines is proportional to the strength of the correlation). A cross-correlation matrix of all active neurons was constructed using the Jaccard correlation coefficient (Fig. 2F) calculated before and during muscarine exposure.

Remarkably, cholinergic modulation enhanced correlated firing (Fig. 2, C–F; 35 lines in NMDA vs. 64 lines in muscarine) without significantly increasing the number of active cells (34 cells in NMDA vs. 39 cells in muscarine). However, not all neurons with correlated firing participated in the peaks of synchronous activity (black vs. red circles), suggesting that correlated firing is not a sufficient condition to affirm that a neuron belongs to a cell assembly (Carrillo-Reid et al. 2008). To compare the actions of the exogenous agonist (muscarine) with that of endogenously released acetylcholine, the acetylcholinesterase inhibitor eserine (10 μM) was used to enhance the basal tone of striatal acetylcholine (see Supplementary Fig. S11). Eserine increased the occurrence of peaks of synchrony and correlated firing in all experiments (n = 12 slices). The increase in neuronal synchronization was mediated by muscarinic receptors because bath addition of atropine (5 μM) decreased the synchronization and returned network dynamics to the control level (NMDA, preeserine; NMDA: 2.3 peak/epoch, 37 cells, 36 correlation lines; eserine: 12.5 peak/epoch, 39 cells, 58 correlation lines; atropine: 2.8 peak/epoch, 36 cells, 33 correlation lines; Supplementary Fig. S1). In summary, the experiments demonstrate that striatal activation of the cholinergic system induces the recruitment of more neurons into correlated firing without increasing the number of active neurons.

A cardinal signature of cell assembly dynamics is that an excitatory tonic drive can be transformed into patterned activity. Patterned activity is characterized by the emergence of various neuronal pools with synchronous, alternating and recurrent activity (Carrillo-Reid et al. 2008; Grillner 2006; Hammer 2003; Harris 2005; Hebb 1949). Based on the results described in the preceding text, we hypothesized that the cholinergic system may endow network dynamics with more complex properties than those conferred by NMDA alone (Carrillo-Reid et al. 2008).

### Cholinergic modulation of striatal network dynamics

To study the actions of the cholinergic system on network behavior, movies were obtained at different time intervals during long time periods (≥1 h; n = 15 slices), and neuronal activity was vectorized (see METHODS) by binning raster plots from all recorded neurons (Carrillo-Reid et al. 2008). The same results were obtained with different bin widths (see METHODS). The vectorization method allowed a rigorous comparison of synchronous peaks before and during muscarine application (Brown et al. 2005; Sasaki et al. 2007). The inner product of all vector pairs, plotted as a matrix, reflected the similarity between all network states appearing in one experiment as a function of time and pharmacological manipulations (Fig. 3A). The appearance of cluster-like structures distributed along time in the similarity index matrix (Fig. 3A) readily suggested radical changes in network dynamics after muscarine addition (Carrillo-Reid et al. 2008; Sasaki et al. 2007; Stopfer et al. 2003).

In confirmation of that assertion, LLE disclosed the presence of various neural vectors representing the different network states (Brown et al. 2005; Carrillo-Reid et al. 2008; Roweis and Saul 2000; Stopfer et al. 2003) (Fig. 3B, see METHODS). Hard and fuzzy clustering algorithms taking the Dunn’s index as a validity function made it possible to depict the different states of the network and the alternation of activity among them over time (Bezdek et al. 1997; Carrillo-Reid et al. 2008; Sasaki et al. 2007). Here data points represent vectorized synchronous peaks and clusters of data points represent the vectors with similar dynamics appearing during recording time. Arrows connecting clusters of data points indicate sequences of activation among pools with synchronously active neurons. It is seen that activity travels among the different network states present within the field of observation. Neuron pools could be seen alternating their activity following recurrent closed pathways and subpathways (Hammer 2003). These dynamics differ greatly from that induced by NMDA alone, both in the number of synchrony peaks and the predisposition of the network to leave one state and readily switch from one to the other (cf., Carrillo-Reid et al. 2008). Figure 3C shows the time course of synchronous peaks occurrences (columns with dots of the same color, each number on the top represents a different 3-min sample movie separated by vertical lines; see METHODS). A representative histogram of overall activity (Fig. 3C, bottom) shows the percentage of coactive cells as a function of time and experimental condition.

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**FIG. 3.** Generation of alternate pathways between network states. A: similarity indices of all vectors representing network states as a function of time. B: multidimensional reduction of vectors representing network states using locally linear embedding (LLE). Each data point represents one vector at a given time projected in the vectorial space delimited by the 1st 2 dimensions. Clusters of vectors represent a given network state. Arrows represent transitions of activity from 1 state to another. Percentages indicate the probability of leaving a given network state. C: raster plots and time histograms of network dynamics. Rows represent the activity of individual cells. Repeating peaks of synchronous activity are marked with asterisks. Vertical lines separate different series of images. First series corresponds to network activity induced by NMDA alone, while the next 2 series correspond to the NMDA-induced activity in the presence of muscarine in the bath. Colors denote different peaks of synchrony corresponding to different network states. Sequential, alternating and recurrent activation of network states can also be noticed in the raster plot. D: spatial maps of the neuron pools from which network states arise (filled circles). Empty contours represent the neurons without activity in the epoch. E: plot of the percentage of coactive cells during different network states. F: hierarchical cluster analysis of cells belonging to the neuron pools underlying the network states (n = 71 cells). Colored boxes indicate cells participating in the different states. A core of neurons shared by all network states is indicated (n = 11 cells).
Figure 3D shows the spatial distribution of each neuron pool representing the different network states. The percentage of coactive cells during the different states is shown in Fig. 3E. Hierarchical cluster analysis of these data revealed the existence of identifiable cells belonging to different and intermingled neuronal pools participating once and again in the network.
activity (Fig. 3F). Interestingly, following the activation of muscarinic receptors, neuron pools were divided into subgroups generating more pathways for the traveling activity, suggesting the property of “distributed synchrony” (Levy et al. 2001). Enhancement of the cholinergic tone also produced more elements that were shared by the different network states (Fig. 3E; 22 ± 2% of shared neurons), as compared with NMDA alone (12 ± 2% of shared neurons; see Fig. 7E in Carrillo-Reid et al. 2008).

These experiments demonstrate that under the present conditions, muscarinic receptor activation led to a fundamental change in the network dynamics of the striatal circuitry: both the level of synchrony and the traveling of the synchronous activity between neuron pools were greatly enhanced. These changes in network dynamics could be theoretically interpreted on a simple basis: they could represent a combinatorial increase in storage capacity during memory processes (Hammer 2003; Luczak et al. 2007; Roudi and Latham 2007), or the increased capability to recruit neurons and to switch between network states during possible related tasks. We next asked if the traveling of synchronous activity from one state to another followed fixed trajectories, that is, if cell assemblies could fire, as expected for Hebbian-like cell assemblies, in a cyclical manner, as do some recurrent neural networks (Abeles 2003; Harris 2005; Hebb 1949; Levy et al. 2001).

Enhancement of cholinergic activity promotes the organization of striatal assemblies into closed cycles

Figures 4A, 1–3, shows the activity patterns during different pharmacological conditions. Eserine and muscarine treatment produced alternating activation of vectors of network dynamics (n = 11/15 slices; see also Supplementary Fig. S2). Also LLE showed an increase in the generation of alternate pathways or closed cycles during cholinergic system activity as compared with the NMDA-alone condition (cf., Fig. 4B, 1–3). Because the time course of activity trajectories during network states appeared to exhibit patterns repeated in time, we identified sentences to follow the changes of the network states over time better (Lee and Wilson 2002) (see black numbers, Fig. 4C, 1–3). Each sentence was constructed as a set of the changes of the network states over time. Sentences were analyzed looking for fixed trajectories or closed cycles. Each closed cycle, which began and ended in the same state, denotes a “word” (sequence of numbers, e.g., “14321”), belonging to a given sentence. Different sequences could be associated with memory traces (Lee and Wilson 2002), but the same sequences may occur on different time scales (Ikegaya et al. 2004; Lee and Wilson 2002). Different words are represented as colored number sequences. The same color denotes the same sequence but beginning at a different point. Fixed sequences were identified with some of them returning several minutes after a previous activation (e.g., Fig. 4C2: “13241” and “24132”; C3: “2412” and “1241”).

We used graph theory to analyze the pathways formed by the network transitions. Each state could be represented as a vertex of a digraph (isomorphic directed graph; see METHODS), and each trajectory could be represented as a directed edge of the digraph (Fig. 4D) (Gerber 1990). Accordingly, two digraphs, G and H, are isomorphic if a function α: V_G → V_H exists such that all vertices of digraph G have a unique correspondence with all vertices of digraph H (Fig. 4D). Isomorphic graphs possess the same theoretical properties and generate the same plane figures (Diestel 2005). We represented segments of state transitions as digraphs labeled with their corresponding words. Digraphs have adjacency matrices (see METHODS, and Supplementary Fig. S3). The representation of digraphs as matrices allows their study under the linear algebra workspace (Gerber 1990). Closed cycles have been proposed as a network mechanism to store memory traces, and these sequences should represent the most efficient memory pathways (Hebb 1949). Thus we chose digraphs that satisfied the conditions of Hamilton or Euler cycles, that is, the best pathways among a fixed set of points (see METHODS, and Supplementary Fig. S3). Figure 4D, 1–3, shows digraphs (directed graphs; see METHODS) composed after the enhancement of cholinergic activation that met this criterion (either eserine or muscarine treatment). Notice that during eserine or muscarine treatment, there is an overlapping of different cycles. It was observed that the conformation of complex cycles from basic ones followed specific rules (n = 35/50 videos; Fig. 4C, 2 and 3; see also Supplementary Fig. S2), demonstrating compositional properties (Bienenstock and Geman 1995; Hammer 2003) of striatal cell assemblies (see following text).

Enhancement of cholinergic tone induces compositionality of striatal cell assemblies

Compositionality is a central issue to cognition; it refers to the ability of a given system to build complex hierarchical representations in terms of simpler parts and their relations (Bienenstock and Geman 1995). There are several systems that have compositional properties, such as language or specific motor actions (Bienenstock and Geman 1995; Grillner 2006; Hammer 2003). Organization of cell assemblies into hierarchical lattices may be an important feature in storing and retrieving any complex knowledge (Hammer 2003), allowing the representation of composite objects by binding simpler components (Abeles 2003; Bienenstock and Geman 1995).

Using graph-based hierarchical conceptual clustering (Jonyer et al. 2001), we showed that striatal cycles can be decomposed into smaller units that can be taken as semantic atoms (Abeles 2003; Hebb 1949). Figure 5 illustrates a lattice of different cycles and their composition rules (colored graphs...
from Fig. 4D2), that we termed cyclic folds. Cyclic folds denote a coordinated neuronal activity that is organized hierarchically. It is formed by activity cycles that fold and unfold (transient events) to compose more complex cycles that may be slightly different each time. Therefore co-activation of cyclic folds represents a complex semantic entity. The existence of smaller units allows the composition of various complex cycles that could be composed following different pathways each time.
Coexistence of several cyclic folds suggests that striatal cell assemblies can support parallel processing or background activation of activity patterns. Activity in the lower levels of the system could initiate activity at higher levels (Abeles 2003; Hebb 1949) endowing striatal cell assemblies with the property of priming. Priming of striatal closed cycles

Because sequential transitions between states could follow closed cycles (such as "14231"), the present activity strongly suggests the existence of mechanisms that determine specific time relations among the firing of cells belonging to different states (Dragoi and Buzsaki 2005; Harris 2005). This form of organization would tend to activate the same combination of cells selectively over time (Hebb 1949), a property called priming in the psychological literature (Abeles 2003). To test if striatal cell assemblies can be primed, we asked whether background activity arising from individual cells exhibits closed cycles outside the peaks of synchrony but similar to those being exhibited among neural vectors. If these background closed cycles were encountered, in much smaller segments of the circuit (before they appear within the synchrony peaks; Fig. 6), then, priming of cell assemblies by parallel low-level processing should be possible. In fact, low-level cycles of activity were easily identified as nested inside segments of different epochs (Fig. 6, A and B) and at different time scales (Lee and Wilson 2002). These specific patterns could be found to run simultaneously in small sets of individual neurons before they appeared within a cycle among synchrony peaks, demonstrating the possibility of low-level parallel processing. Indeed cycles shown in Fig. 6B did not participate in the peaks of synchronous activity (dashed box from Fig. 6A) until 10 min later. This implies that reverberating activity may synchronize and desynchronize different cell assemblies over time, allowing the appearance of the same words during state transitions depending on the past states (internal states) of the network. It has been proposed that multiple parallel or alternate processing of information could be a useful mechanism to provide resilience in the face of brain damage (Hebb 1949).
neurons can enter and depart from the synchronized states giving rise to network states. In fact, an increased number of traveling of synchronized activity among the neuron pools increased correlated firing was accompanied by a more efficient voltage transitions. These effects are translated into a significant increase in the correlated activity of the network. 2) Increased correlated firing was accompanied by a more efficient traveling of synchronized activity among the neuron pools giving rise to network states. In fact, an increased number of both spontaneous peaks of synchrony and trajectories in the flow of activity were evident. These results demonstrate that neurons can enter and depart from the synchronized states modulated by cholinergic tone. 3) A closer analysis of activity trajectories revealed recurrent sequences of activation of neuron pools. Alternative trajectories for the activity following a set of pathways and subpathways revealed that the cholinergic system induces cell assemblies with compositional capabilities (Bienenstock and Geman 1995; Hammer 2003). These findings suggest that the interface between neuronal activity and circuitry function can be approached (Graybiel 1995).

DISCUSSION

We used dynamic multi-cell calcium imaging of neuronal populations and whole cell voltage recordings from neostriatal spiny neurons, in combination with analytical approaches, to demonstrate that: 1) muscarinic receptor activation facilitates both the negative slope conductance region of the current-voltage relationship induced by NMDA and the generation of voltage transitions. These effects are translated into a significant increase in the correlated activity of the network. Basic modules or semantic atoms (Abeles 2003; Hebb 1949) could be thought as “unit central pattern generators (CPGs),” that is, a group of neurons generating recurrent activity (Grillner 2006). The nervous system is endowed with a number of inborn or preassembled unit CPGs that can be recruited to generate a rich variety of behaviors (Carrillo-Reid et al. 2008; Grillner 2006). However, while NMDA only induces a feedback activity more similar to that encountered in unit CPGs (Carrillo-Reid et al. 2008; Grillner 2006), cholinergic modulation endows striatal cell assemblies with a more interesting property: compositionality (Bienenstock and Geman 1995; Hammer 2003).

Actually, the ability of neuronal pools to compose complex and synchronized sequences of activity is the main difference between our findings and those from other groups working in the cortex. Thus synfire chains are based on the sequential activation of individual cells (Ikegaya et al. 2004), while our results are based on the synchronous and sequential firing of neural vectors over time. These neural vectors form cycles of activity that are organized hierarchically with a modular architecture: larger cycles are composed from simpler ones. Moreover, activity of closed cycles can be repeated exactly in the same order. To look for priming sequences in individual cells, we considered the onset of the calcium signals (arrows); this indicates the state of a given cell to be considered. The sequences “2142,” “1421,” and “1241” appeared as in state transitions from peaks 37 to 49. Those “words” were found before they appeared in the peaks of synchronous activity. Note synchronous activity of cells 12 and 14.

Building composed microcircuits

It is thought that complex motor actions can be recursively decomposed into simpler motor actions (Bienenstock and Geman 1995; Grillner 2006). These basic units can be recombined to generate a diverse and flexible output (Grillner 2006). Alternative trajectories for the activity following a set of pathways and subpathways revealed that the cholinergic system induces cell assemblies with compositional capabilities (Bienenstock and Geman 1995; Hammer 2003). These findings suggest that the interface between neuronal activity and circuitry function can be approached (Graybiel 1995).

A central issue in neuroscience is how neural networks store memories. Hebb (1949) proposed reverberant cell assemblies as the substrate for memory storage. However, to store any kind of composed knowledge, cell assemblies must have the ability to form hierarchies: subcircuits composing larger circuits (Hammer 2003). Composition refers to hierarchical construction of representations made up of simpler parts and their relationships (Bienenstock and Geman 1995). A realistic memory system must be composed of interconnected modules; such a modular structure can be the basis for a combinatorial increase in storage capacity (Huyck 2001).

Among the two Hebbian hypotheses concerning memory processes, the one concerning long-term synaptic plasticity has received exhaustive experimental support following the work of Bliss and Lomo (1973), while the one concerning the existence of cyclic reverberant cell assemblies or recurrent neuronal networks (Hammer 2003) has proved more elusive. Here we recorded neuronal microcircuits that clearly exhibit recursive cell assembly properties in the rat neostriatum in vitro. The methods used allowed us to follow multiple cells simultaneously in a small segment of the network. The activity of similar neuron pools has been recorded in the cortex and other brain structures (Cossart et al. 2003; Ikegaya et al. 2004), but previous analysis stopped short of demonstrating the recurrent and reverberating nature of neural activity.

From cell assemblies to memory processes

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metastability found in hippocampal CA3 networks (Sasaki et al. 2007).

Organization of recurrent closed cycles of activity

The demonstration of the sequential and recurrent traveling of synchronous activity among network states reveals a robust and coordinated activity of specific, identified neuron pools. These pools are intermingled within (Cossart et al. 2003; Hebb 1949; Ikegaya et al. 2004) a small area of an in vitro slice preparation. The recursive activation of spatiotemporal patterns following closed cycles of recurrent activity was hypothesized when looking at histological preparations of the same size scale (Hebb 1949; Lorente de No 1938). It has also been inferred from electrophysiological recordings of patterned activity of small numbers of neurons during simultaneous field recordings (Abeles 2003; Beiser et al. 1997; Berke et al. 2004; Costa et al. 2006; Dragoi and Buzsáki 2005; Luzak et al. 2007). This activity has been posited as a plausible mechanism to store memories, including procedural memories (Abeles 2003; Beiser et al. 1997; Hammer 2003). Because the ability to record from dozens of cells simultaneously has only recently been available (Cossart et al. 2003; Ikegaya et al. 2004; Mao et al. 2001), it perhaps should not come as a surprise that the finding of these capabilities in small segments of tissue in vitro has appeared only after a relatively long time since the advent of the brain slice preparation.

Mechanisms of striatal activity cycles

We demonstrated previously that the NMDA-induced network dynamics reside in both the synaptic and intrinsic properties of striatal neurons. Blockade of GABAergic transmission disrupts alternation and selection among different neuron pools demonstrating that inhibitory connections in the striatum are essential for the recursive activity of striatal cell assemblies (Carrillo-Reid et al. 2008; Ossowska and Wolfarth 1995). Furthermore, a tonic excitatory drive elicited by NMDA application to neostriatal microcircuits generates both bursting activity (Carrillo-Reid et al. 2008; Vergara et al. 2003) and motor behavior (Ossowska and Wolfarth 1995), demonstrating that a physiological significant mechanism is preserved in vitro. The striatum has been posited as the store of some inborn motor circuits (CPGs) (Grillner 2006) and also of learned or acquired ones (Aosaki et al. 1994; Diaz del Guante et al. 1991; DeLong et al. 1986; Graybiel 1995). In fact, network dynamics have been shown in both young (PD13-22) and adult (PD >40) animals (Carrillo-Reid et al. 2008; Ikegaya et al. 2004; Lee and Wilson 2002) demonstrating that network activity can be observed over a wide range of ages.

However, not every increase in the excitability of the network results in the same behavior. Thus blockade of GABAergic transmission increases the number of synchrony peaks but abolishes network dynamics. On the other hand, increases in NMDA concentration do not change the dynamics produced by this transmitter alone (Carrillo-Reid et al. 2008). And finally, selective cholinergic antagonists reverse the dynamics induced by muscarinic to that found just with NMDA.

In summary, activation of the cholinergic system engendered synchronized activity that traveled in closed cycles with a modular structure and a hierarchical organization (Abeles 2003; Beiser et al. 1997; Grillner 2006; Harris 2005; Hebb 1949; Lewis et al. 2005; Roudi and Latham 2007). It is intriguing, that this activity involves circuitry composed mainly of inhibitory neurons.

Functional implications

While the present work aimed to extend our understanding of normal striatal physiology, it may also have significant implications for the elucidation of pathological conditions. It is known that acetylcholine levels are increased in Parkinson disease and that anticholinergic drugs are frequently administered as adjuvant therapy with inconsistent results (Pisani et al. 2007). Some theories about Parkinson’s disease suggest the appearance of excessive synchrony in the basal ganglia (Beiser et al. 1997) brought about in part by a chronic elevation of cholinergic tone due to dopamine loss (Pisani et al. 2007). The chronic elevation of synchronous activity observed could engage fixed neuronal assemblies, thus discontinuing the association of different modules and restricting compositional capabilities (Levy et al. 2001). Thus network dynamics and the role of intrinsic and synaptic mechanisms involved in the abnormal synchronization of firing patterns in pathological states deserve further study with the approaches we have used.

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