Video Article

Induction of an Isoelectric Brain State to Investigate the Impact of Endogenous Synaptic Activity on Neuronal Excitability *In Vivo*

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Abstract

The way neurons process information depends both on their intrinsic membrane properties and on the dynamics of the afferent synaptic network. In particular, endogenously-generated network activity, which strongly varies as a function of the state of vigilance, significantly modulates neuronal computation. To investigate how different spontaneous cerebral dynamics impact single neurons' integrative properties, we developed a new experimental strategy in the rat consisting in suppressing *in vivo* all cerebral activity by means of a systemic injection of a high dose of sodium pentobarbital. Cortical activities, continuously monitored by combined electrocorticogram (ECoG) and intracellular recordings are progressively slowed down, leading to a steady isoelectric profile. This extreme brain state, putting the rat into a deep comatose, was carefully monitored by measuring the physiological constants of the animal throughout the experiments. Intracellular recordings allowed us to characterize and compare the integrative properties of the same neuron embedded into physiologically relevant cortical dynamics, such as those encountered in the sleep-wake cycle, and when the brain was fully silent.

Video Link

The video component of this article can be found at <http://www.jove.com/video/53576/>

Introduction

In the absence of any environmental stimuli or behavioral tasks, the "resting" brain generates a continuous stream of electrical activity that can be recorded from the scalp, as electroencephalographic (EEG) waves. The intracellular correlate of this endogenous cerebral activity is characterized by background membrane voltage fluctuations (also known as "synaptic noise"), which are composed of a combination of excitatory and inhibitory synaptic potentials that reflect the ongoing activity of afferent networks^{1,2}. This spontaneous activity varies in frequency and amplitude with the different states of vigilance. Elucidating the impact of network activity on the excitability and responsiveness of single neurons is one of the major challenges of neurosciences^{3,4}.

Many experimental and computational studies have explored the functional impact of ongoing synaptic activity on the integrative properties of neurons. However, the role of the different neuronal parameters impacted by the background synaptic noise remains elusive. For instance, the mean level of membrane depolarization has been found positively^{5,6} or negatively⁷⁻⁹ correlated with the ability of sensory inputs to trigger action potentials. Moreover, whereas some investigations suggest that fluctuations of the membrane potential, resulting from a continuously varying stream of afferent synaptic inputs, strongly affect the responsiveness of single neurons by modulating the gain of their input-output relationship^{3,10-13}, others indicate that changes in membrane input conductance mediated by shunting inhibition are sufficient to modulate the neuronal gain regardless of the magnitude of membrane fluctuations^{14,15}. Finally, recent studies performed on awake animals stressed how the processing of sensory information in single neuron critically depends upon the state of vigilance and the current behavioral demand^{16,17}.

A straightforward strategy to elucidate the functional role of a given process in a highly interconnected system is to determine how its absence specifically alters the functioning of the system. This method has been extensively used in neuroscience research, for example using experimental lesions or inactivation of different brain areas¹⁸⁻²¹, or pharmacological blockade of specific ion channels^{22,23}. Notably, it has been applied *in vivo* to unveil how functional connectivity and network dynamics affect single cell computation²⁴⁻²⁷. However, to date local manipulations intended to block the firing of neurons and/or perturb their basic biophysical properties can be partially effective and are limited to relatively small brain volumes²⁸.

To overcome these limitations, we developed a new *in vivo* experimental approach in the rat to compare the electrophysiological properties of single neurons recorded in a given brain state, *i.e.*, embedded in a particular network dynamic, to those obtained after complete suppression of the whole brain synaptic activity²⁹. In the control conditions, two distinct cortical dynamics could be generated. Sleep-like electrocorticographic

(ECoG) patterns were induced by injection of moderate doses of sodium pentobarbital. Alternatively, fast ECoG waves of small amplitude comparable to the cortical activity underlying the waking state (waking-like pattern) could be produced by injection of fentanyl. Subsequently, while maintaining the same ECoG and intracellular recording, a complete silencing of endogenous brain electrical activity was obtained by systemic injection of a high dose of sodium pentobarbital, characterized by isoelectric ECoG and intracellular activities. Because the induction of such an extreme comatose could potentially have fatal consequences on biological functions, a careful and continuous monitoring of the physiological variables was essential. Therefore, we meticulously followed the heart beat frequency, the end-tidal CO₂ concentration (EtCO₂), the O₂ saturation (SpO₂) and core temperature of the rat throughout the experiments.

We evaluate single neurons properties during these different states using sharp microelectrodes, which are particularly suited for long and stable recordings *in vivo*. The procedure described here, can be combined with other electrophysiological and imaging approaches and could be extended to other animal models.

Protocol

All procedures were carried out in accordance with the guidelines of the European Union (directive 2010/63/EU) and approved by the Charles Darwin Ethical Committee on Animal Experimentation. We describe here the procedure we routinely use in our laboratory, however most steps can be adapted to match everyone's specific needs.

1. Surgical Preparation

Note: All incision and pressure points should be repeatedly infiltrated with local anesthetic (lidocaine or bupivacaine). The present procedure is terminal, if an aseptic preparation is required several modifications should be implemented.

1. Anesthetize a rat with sodium pentobarbital (40 mg/kg) and ketamine (50 mg/kg) in two locally separated intraperitoneal (IP) injections.
2. Let the animal go under general anesthesia and repeatedly verify that a surgical plane of anesthesia is attained (no reaction to toe pinching). Place the rat on a feedback heating blanket and insert a rectal probe to maintain core temperature around 37 °C.
3. Place a catheter in the peritoneal cavity to facilitate subsequent injection of anesthetic agents and to avoid perforating organs by repeated needle punctures³⁰.
 1. Clip the hair over a small (~2 - 3 mm) area above a region located within the stomach's lower right or left quadrant.
Note: Depilatory cream can also be used.
 2. Make a 2 - 3 mm incision on the skin with sharp scissors or a scalpel. Using blunt dissection, remove fat and muscle layers until the peritoneal cavity is observed.
 3. Insert about 1 - 2 cm of a small catheter in the cavity and close the wound with surgical glue.
Note: The diameter and the total length of the catheter should be minimal (e.g., 2 French - corresponding to 0.043 mm inside diameter) to reduce dead volumes. Polyurethane tubes are most suited.
 4. Make a loop and suture the catheter to the skin to secure it in place.
4. Install a tracheal tube to control ventilation during artificial respiration.
 1. As for the previous step, prepare the area of interest (1 - 2 cm over the trachea right above the manubrium), remove the hair and incise the skin.
 2. Blunt dissect the first layers of fat and muscles, then move the salivary gland aside and expose the trachea by gently dissecting the last layer of muscles.
 3. Carefully remove the tissues over the trachea and slide a thread below it using small forceps. Make a surgeon's knot with the thread but do not tight it yet.
 4. Incise the trachea transversally between two cartilaginous rings. Swab blood in the trachea if any.
 5. Insert a tracheal tube with the appropriate diameter and tighten the thread's knot to secure the tube steady. For additional stability the thread can further be attached at a higher point of the trachea tube.
 6. Suture the wound close or use surgical staples. Avoid surgical glue here if the tracheal tube is intended to be reused.
5. Install the animal in a stereotaxic frame and carefully monitor the following physiological variables: ECoG, SpO₂, EtCO₂, heart rate (via an electrocardiogram, ECG) and internal temperature. Monitor and adjust these variables to keep a proper depth of anesthesia and physiological state. Specifically, supplement anesthesia with a small dose (10 mg/kg) of sodium pentobarbital if necessary.
6. Apply eye ointment on both eyes to avoid desiccation. Clip the hair over the scalp, make a longitudinal incision (~2 cm) and resect the connective tissues overlying the skull using a scalpel or a curette.
7. Make a small (~1.5 mm diameter) craniotomy over the region of interest with a dental drill.
Note: Here, the barrel field of the primary somatosensory cortex is targeted (7 - 8 mm anterior to the interaural line, 4.5 - 5.5 mm lateral to the midline³¹). Rinse repeatedly to dissipate heat.
8. Use extra fine forceps to gently make a small hole in the dura. Reserve a ~0.5 mm region within the cranial trepanation to place the ECoG electrode (see next step). Permanently keep the cortex moist with 0.9% NaCl solution (or artificial cerebro-spinal fluid).
9. Place a low impedance (~60 kΩ) silver electrode (the ECoG electrode) on the dura, avoiding the cortical region not covered by the meninges, and place the reference electrode on a scalp muscle on the other side of the head.
10. At this stage (30 min after the last sodium pentobarbital injection), maintain the anesthesia by repeated injections of sodium pentobarbital (10-15 mg/kg/h) or fentanyl (3-6 µg/kg/h) via the IP catheter. The former will result in a slow oscillatory, sleep-like, ECoG pattern whereas the latter will result in a desynchronized, waking-like, cortical profile.
11. Adjust the artificial ventilation system so that the respiratory frequency and volume are similar to those of the rat's spontaneous breathing (normal range 70 - 115 breath/min³²). Then, connect the mechanical ventilation to the tracheal tube and verify the proper thoracic cage inflation (on both sides). If not, adjust the position of the ventilation tubing in the tracheal axis. If necessary, suck up the secretion present in the trachea with a catheter connected to a syringe or a vacuum pump.

12. If all physiological variables³²⁻³⁵ and ECoG^{29,36} patterns reflect a stable surgical plane of anesthesia, do an intramuscular injection of gallamine triethiodide in each leg to paralyze the rat, 40 mg/kg for the first injection and then 20 mg/kg, every 2 hr^{19,29,36,50}.

2. Intracellular Recordings

1. Pull a glass micropipette (sharp microelectrode) with a $\sim 0.2 \mu\text{m}$ tip such as its resistance ranges between 50 and 80 M Ω once filled with 2 M potassium acetate (KAc).
2. Place the pipette in a specific holder with a silver/silver-chloride (Ag/AgCl) wire to connect the pipette solution to an intracellular amplifier (via a head stage). The holder should be attached to a micromanipulator. Place a Ag/AgCl reference electrode on the rat's neck muscles.
3. Slowly insert the pipette in the brain down to the region of interest and verify its resistance by monitoring the voltage drops in response to current steps. Use the buzz (or zap) button of the amplifier to clear the pipette if needed.
4. Use cotton swabs or synthetic absorption triangles to dry the craniotomy (be careful not to touch the ECoG or intracellular electrodes) before covering it with silicone elastomer or 4% agarose to reduce brain movements.
5. Lower the pipette in 1 - 2 μm steps until its resistance increases when approaching a cell. Then use the buzz function of the amplifier to penetrate into the neuron.

3. Induce the Isoelectric State

1. Perform the appropriate experimental protocol (e.g., firing responses to intracellularly injected currents, current-voltage relations, sensory stimuli responses) at this stage while monitoring simultaneously the neuron's membrane potential, the ECoG and the physiological variables.
2. Ensure that the intracellular electrode is stable in the cell by analyzing the steadiness of both the membrane potential and the action potential properties throughout the recording session. If not, do not go to the next step and wait until the recording becomes stable again or search for another neuron.
3. Inject a high but infra-lethal dose of sodium pentobarbital ($\sim 90 \text{ mg/kg}$, can be as low as 35 mg/kg from the pentobarbital initial condition and up to 155 mg/kg from the fentanyl initial condition) via the IP line.
Note: Within 15 - 20 min the intracellular and ECoG waveforms should slow down with intermittent electrical silences to transiently reach the so called "burst-suppression" profile^{37,38}, which progressively collapses to a complete isoelectric state. It is expected that the heart rate significantly slows down (by $\sim 10 - 20\%$) but the SpO_2 and EtCO_2 should stay relatively steady.
4. If the isoelectric state is not reached, inject a small amount of sodium pentobarbital ($\sim 10\%$ of the step 3.1 dose). Wait 15 min before adding more anesthetic if the isoelectric state is still not achieved.
5. Repeat the experimental protocol to compare the impact of the network dynamics on the recorded neuron's integrative properties.
Note: After cessation of anesthetic injections, the electrical brain activity should fully recover within 3 to 4 hr.
6. At the end of the experiment, inject a lethal dose of sodium pentobarbital (200 mg/kg, IP) to euthanize the rat.

Representative Results

Inducing and maintaining an isoelectric brain state is a delicate *in vivo* experimental procedure. It has been proved to be a powerful tool to directly study the impact of cortical network activity on neuronal excitability and transfer function²⁹. **Figure 1** shows the multi-parameter monitoring, including ECoG and vital constants, of the animal's physiological state before (**Figure 1A**) and after (**Figure 1B**) induction of the isoelectric state.

Figure 1. Monitoring of the Physiological Parameters in Control and Isoelectric Conditions.

A and **B**, Simultaneous recordings of ECoG (top traces) and physiological parameters during active cortical state (**A**) and subsequent isoelectric period (**B**). Core temperature (Temp.), EtCO_2 and SpO_2 are essentially stable throughout the experiment. Heart beat rate, in contrast, progressively decreases following the induction of the isoelectric state (from 382 to 349 beats/min), as seen on the ECG. [Please click here to view a larger version of this figure.](#)

During the control sessions, the physiological parameters were similar to those measured in healthy and awake animals³²⁻³⁵ and remained unaffected after induction of the isoelectric state, except for the heart rate that was slightly slowed down (**Figure 1**). This is an important point since hypoxia³⁹ or hypercapnia⁴⁰ can markedly alter neuronal excitability and thus can introduce a serious bias in a study exploring a brain state-dependent modulation of neuronal integrative properties.

We obtained the isoelectric status from two different initial conditions mimicking the cortical dynamics endogenously-generated during the early stages of sleep (**Figure 2Aa**, left panel) or during waking (**Figure 2Ab**, left panel). These active states were either induced by injection of sodium pentobarbital (sleep-like) or fentanyl (waking-like). In both cases, the subsequent injection of a high dose of sodium pentobarbital resulted in a complete abolition of spontaneous activity in the ECoG and the simultaneously recorded neurons (**Figure 2Aa** and **b**, right panels), hence the term isoelectric. Suppressing the ongoing synaptic activity resulted in a significant steady hyperpolarization of the neuronal membrane potential (**Figure 2B**).

Figure 2. Consequences of Synaptic Activity Suppression on the Spontaneous Dynamics of Membrane Potential. (A) Simultaneous representative recordings of ECoG (top traces) and intracellular activities (Intra, bottom traces) during sleep-like (A_a) and waking-like (A_b) patterns (left panels), and during the corresponding subsequent isoelectric epochs (at the times indicated after the suppressive injection; Isoelectric, right panels). The time-frequency analysis of ECoG signals (energy density for the 0 - 50 Hz frequency range) is depicted by color scales. (B) Probability densities (P) of membrane potential values (Vm, bin size 0.5 mV, 10 sec of recording) from the neurons illustrated in panel A. This figure has been modified, with permission, from ref 29. [Please click here to view a larger version of this figure.](#)

To illustrate the functional impact of this extreme brain state, we extracted the passive and active intrinsic properties of isoelectric neurons and compared them with those measured during the corresponding initial condition. Using this strategy, we have shown that neurons could fire action potentials in response to intracellular injection of depolarizing current during the isoelectric state, demonstrating that they remained fully excitable even after the complete suppression of background synaptic activity (**Figure 3Aa** and **b**, Isoelectric). Moreover, we found that the transfer function of neurons, assessed by measuring the firing frequency induced by steps of depolarizing current of increasing intensity (F-I relationship), was right-shifted compared to the initial active conditions, indicating a decrease in the sensitivity of neurons to weak excitatory inputs (**Figure 3B**). The corresponding neuronal gain, *i.e.*, the slope of the F-I curve, remained unchanged or was reduced when the control state was of sleep- or waking-type, respectively (**Figure 3B**). Surprisingly, the apparent input resistance of neurons was not significantly modified in the absence of synaptic drive compared to the control active conditions (**Figure 3Aa, b**). More results, including population analysis and quantification of the temporal firing patterns of neurons in the active and isoelectric conditions, are available in our initial paper ²⁹.

Figure 3. Comparative Impact of the Three Cortical Activity Patterns on Membrane Properties and Input-output Relations. (A) Voltage responses (middle traces) of somatosensory cortical neurons to depolarizing and hyperpolarizing current pulses (bottom traces) during sleep-like (Aa) and waking-like (Ab) ECoG patterns (top records) and after deprivation of synaptic activity (Isoelectric). Membrane input resistance (Rm, values are indicated) was measured from voltage drops (grey traces, averages of 20 trials) induced by hyperpolarizing current pulse injections (-0.4 nA). (B) Corresponding F-I curves, providing the transfer function of the neurons illustrated in panel (A). The firing rate was measured in response to depolarizing current pulses (200 msec duration) of increasing intensity. Each current intensity was applied 20 times and the corresponding firing rates were averaged. The dashed lines indicate the cell responses shown in (A). This figure has been modified, with permission, from ref 29. [Please click here to view a larger version of this figure.](#)

Discussion

We describe here a new method to suppress *in vivo* spontaneous cerebral electrical activity at both network and cellular levels. This procedure leads to an extreme brain state, known as isoelectric comatose⁴¹. From a clinical point of view, such an electrocerebral inactivity is the most severe abnormality that can be seen on the EEG. It is mostly associated with an irreversible coma, with all patients either dying or continuing in a persistent vegetative state⁴², but can be at least partially reversed when caused by an intoxication with central nervous system depressant medications (such as thiopental), an accidental hyperthermia⁴² or an asphyxial cardiac arrest⁴³. In our experimental paradigm, isoelectric state is progressively achieved by a systemic injection of sodium pentobarbital at high doses, which first rapidly induces a reduction in the ECoG frequency content, then a "burst-suppression" mode^{41,42}, leading finally to a completely flat ECoG. At the intracellular level, the disappearance of spontaneous activity follows a similar time course with concomitant reduction of depolarizing and hyperpolarizing membrane potential fluctuations. Thus, it can be hypothesized that the injection of sodium pentobarbital first increases the synaptic inhibitory transmission leading to a reduction of cortical neurons firing activity, the progressive abolition of excitatory and inhibitory synaptic transmission that finally results in isoelectric ECoG and intracellular activities^{29,44}. Similar transitions from active to isoelectric ECoG patterns can be obtained following the administration of other anesthetic agents such as ketamine (personal observation) or isoflurane^{45,46}.

The procedure may appear relatively straightforward. However, due to the extremely deep comatose induced, maintaining the basic physiological variables within normal ranges is of paramount importance for the success of the experiment. Changes in EtCO₂ fluctuations may be the result of a mucus plug forming in the trachea. In such a situation, the ventilator should be disconnected and the mucus quickly aspirated or wiped out through the tracheal tube. Moreover, the mechanical stability of the preparation is crucial for intracellular recordings. Thus, special efforts should be made to reduce vascular and respiratory pulsations, by carefully adjusting the animal's body relative to the head while maintaining a proper tracheal tubing alignment, and by applying agarose or silicone elastomer on the craniotomy. It is also necessary to avoid spontaneous muscle contractions by injection of a paralyzing agent. Finally, environmental vibrations and electrical noise should be reduced as much as possible. Other publications detail the essential steps for an optimal *in vivo* preparation allowing stable intracellular or patch-clamp whole-cell recordings^{29,47-50}.

The ability to decouple neuronal intrinsic membrane properties and networks dynamics is essential to dissect the mechanisms by which individual neurons process information in their highly connected environment. As stated in the Introduction, previous studies devoted to this

central issue of fundamental neurosciences led to conflicting results, partly due to the specific features of neurons and networks investigated and the various experimental conditions, including *in vitro* vs *in vivo* preparations and, eventually, the different anesthetic procedures used (see for instance^{29,36,51}). We propose that the present approach could be used to validate, and possibly reconcile, findings obtained from the reduced *in vitro* preparation and from *in vivo* experiments. Indeed, it allows to directly examine and compare in the same neuron and in the course of the same experimental procedure the impact of distinct patterns of afferent synaptic inputs, from waking-like dynamics to complete inactivity, on the neuronal integrative properties in a living animal.

One distinctive feature of this protocol is that, once mastered, it could be combined with other experimental techniques, such as multisite surface and depth EEG recordings, genetically encoded fluorescent indicators based investigations and even hemodynamic and metabolic imaging of the brain, to investigate the multidimensional properties of the isoelectric brain. As clinical and diagnostic perspectives, since we demonstrated that neurons are still excitable during persistent isoelectric comatose, it would be relevant to test the cortical functions, for example the processing of sensory information, of patients and animal models immersed in such a pathological state of brain inactivity.

Disclosures

The authors have nothing to disclose.

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