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Neuronal Activity

From In Vitro Preparation to Behaving Animals

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Abstract

The knowledge of the mechanisms regulating electric neuronal activity is fragmented by the wide variety of techniques and experimental models currently used in neurophysiological research. The interest and importance of the results obtained in any research is improved when interpreted in the perspective of the organism functioning as a whole in physiological conditions. Such interpretation, freed of the constraints imposed by the different techniques and experimental conditions used, is especially important when discussing together results obtained at the behavioral, cellular, and molecular level. This article outlines some of the key factors to consider when experiments from different models are interpreted together.

Index Entries: In vitro; slice preparation; in vivo; anesthesia; freely moving; experimental models.

Introduction

Although the understanding of brain processes has been augmented by more detailed and precise studies, assimilation of this newly acquired information into a perspective of brain function has become more difficult. This problem is not new or specific to neuroscience. Claude Bernard, the pioneer of experimental research in physiology acknowledged that, "If we break up a living organism by isolating its different parts, it is only for the sake of ease in experimental analysis, and by no means in order to conceive them separately. Indeed when we wish to ascribe to a physiological quality its value and true significance, we must always refer it to this whole, and draw our final conclusion only in relation to its effects in the whole" *(1)*.

This necessary reconciliation between focused studies conducted in isolated, simplified preparation and work realized in more physiological conditions is obviously interesting but is also a challenging task.

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Among the early attempts to study the intact brain, recording the electrical signals it generated played a major role. Electroencephalography allowed the description of rhythmic activity of the nervous system, even if the identification of the source of the signal detected was a matter of speculation at that time *(2–5)*. The development of more sophisticated tools later enabled study of the electrical signal at the scale of the neuron. These progressions from the macroscopic and global level to the molecular and singular phenomenon led to the isolation of currents generated by single channels involved in the generation of neuronal electrical activity *(6)*. It is now possible to study the molecular mechanisms underlying the electrical activity of neurons as well as fluctuations of firing rates in several areas of the brain in behaving animals with different experimental approaches; each approach has its own advantages and limitations. Experimental results from these studies represent the different levels to which the brain may be studied (from ion channels to behavior-specific neuronal activity). However, using the results obtained at one level to interpret experimental data from another level remains a difficult challenge. Important factors may limit the comparison of results obtained in the different preparations used in neurophysiological studies (i.e., in vitro slice preparation, anesthetized animals, or freely moving animals). This article reviews some of these factors and their impact on the original interpretation of the results in the original experimental context and at subsequent levels of complexity in other preparations.

First, experimental data from behaving animals are presented and discussed. This preparation may be considered a reference point because it best mimics physiological conditions. In fact, fundamental mechanisms of neurotransmission, such as the action of glutamate (GLU), appear to be different when studied in awake and anesthetized animals *(7)*. Although awake and behaving animals offer the best conditions for the study of brain neurophysiology, this method also has considerable drawbacks. This article reviews and considers some of the limits of this model, their impact on the results obtained, and their interpretations.

Anesthetic agents are widely used in neurophysiological studies; they provide a quiet, stable state that allows work at neuronal scale in relatively physiological conditions. Beyond their specific mechanisms of action, which may account for some differences observed between studies, other physiological parameters can be out of balance under anesthesia and, therefore, may influence the outcome of the research. This article reviews the effects of some anesthetic agents on neuronal excitability and then presents the parameters most likely altered by anesthesia, which thereby modify neuronal electrical activity.

Numerous in vitro studies—specifically those using slice preparation—largely contributed to the understanding of neurophysiology. This method has many advantages, including control of the composition of the recording medium, and provides mechanical stability for long-term intracellular recording. Another advantage is the isolation of the neurons contained in the slice from the rest of the brain; this makes the results obtained specific to the cell studied and free of afferents' influence. However, this can become an important concern if the work is viewed in a more global context. Nevertheless, when discussing results from brain slices, the loss of structural integrity also must be questioned *(8)*; more recently, the influence for in vitro preparations of other parameters, such as temperature or morphological re-arrangements, have been emphasized *(9–12)*.

Freely Moving and Head-Restrained Animals

Background

Neuronal activity can be studied in freely moving or unanesthetized, head-restrained animals. These preparations offer unique opportunities to work in physiological conditions, without the interaction of anesthetic agents *(13,14)*. Because animals are fully exposed to

environmental variations and are also subject to their normal biological rhythms in these situations, long-term, stable conditions are difficult to maintain. Other factors such as housing conditions, age and gender of animals used, or diet may have an unexpected influence on spontaneous or induced neuronal activity.

Circadian Cycle

In the course of a day, laboratory animals go through a circadian cycle entrained by the alternation of the light and dark phases (for review, *see* ref. *15*). Based on the pattern of activity of the electroencephalogram and electromyogram, it is possible to separate the 24-h period in different phases (i.e., awake, slow-wave sleep [SWS] and paradoxical sleep [PS]).

The suprachiasmatic nucleus is proposed to be the central pacemaker that regulates the circadian cycle *(16)*; this rhythmic activity appears to be controlled by a γ-aminobutyric acid (GABA)-dependent mechanism *(17)*. Recently accumulated experimental data tend to prove that this structure is not the only circadian oscillator in the brain *(18)*. The interest here is not an extensive discussion on the origins of circadian rhythm but, rather, to consider the consequences of this cycle on neuronal activity, even if separating the origins and results of such a global biological process is difficult. Transitions from waking to sleep are mirrored by changes in the expression of specific genes *(19)*. This is translated by circadian variations in the expression of various receptors such as type 1 cannabinoid receptors with a protein expression maximum at 13H00 and minimum at 1H00 *(20)*. Circadian variations for the expression of the receptor to brain-derived neurotrophic factor have also been observed *(21)*. The same variations in protein expression probably are involved in the daily changes of muscarinic binding observed in hypothalamus and hippocampus *(22)*.

Neurotransmitter levels are also subject to variation during the day. In striatum and Nucleus Accumbes, GABA, GLU, and dopamine (DA) extracellular contents vary following a circadian cycle. In Nac, these variations are not influenced by animals' exposure to light, whereas in striatum, only the DA level changes following the pace of the light–dark cycle *(23,24)*. In prefrontal cortex, serotonin (5-HT) concentration can increase up to 450% during the wake cycle compared to SWS, whereas DA and its metabolites decrease during the wake cycle compared to SWS. For PS, variation of 5-HT and DA depend on which phase follows the episode *(25)*. Higher content of nitric oxide was also observed during waking compared to SWS and PS *(26)*. These variations following the different phases of the wake–sleep cycle were observed for other neurotransmitters or neuromodulators in various brain areas and in different species *(27,28)*.

Variations of neuronal activity and their correlation with different phases of the wake–sleep cycle have long been studied *(29)*. Across this cycle, the same cell can switch between different levels of activity and can go through episodes of silence *(30)*. Numerous cell populations can be individualized within the same area based on their activity in the different phases of the circadian cycle *(13,31–34)*. Both the average firing of neurons and their patterns of activity can change within the different phases of the wake–sleep cycle. For example, in the subthalamic nucleus, neurons switch from a random activity observed during the wake cycle to bursts observed in SWS *(35)*.

Together, several of these variations are probably involved in the different evoked responses observed during the wake–sleep cycle. For example, the nicotine-induced release of GLU in cerebellum presents a diurnal variation *(36)*. Differences in stimuli-evoked response in several areas of the hippocampus were also observed between the phases of the wake–sleep cycle *(37)*. Notably, even under anesthesia, some circadian variations in neurotransmitter-evoked response are preserved *(38)*.

Considering the important changes in neuronal activity that follow the circadian oscillation, it appears critically important to compare results obtained in similar phases of this cycle. This can probably be achieved by maintaining

a strict light–dark cycle and precisely documenting the time at which experiments are performed during the day. However, aversive or rewarding stimuli can shift the phase of the circadian cycle, thus undermining comparisons of match-phase results based on the assumption that the light–dark cycle drives the circadian rhythm. However, certain species of rodents appear particularly resistant to photic-phase shift *(39)*.

Circadian Cycle and Aging

The circadian cycle is subject to age-related changes that affect the phases of the wake–sleep cycle differently *(40–43)*. This article previously discussed the influence of the circadian cycle on neuronal activity and some factors that affect it; this section presents the effects of aging on these wake–sleep-related variations. The amplitude of nitric oxide release in the cortex that follows the wake–sleep cycle is higher in older animals *(44)*. Conversely, aging decreases the diurnal changes in prefrontal acetylcholine (ACh) release. This ACh level is related only to motor activity in young animals compared to older animals *(45)*. In the hippocampus, the electrophysiological activity of neurons is bursts with less impact during the wake cycle in older animals, and the burst frequency decreases *(46)*. In ventral tegmental nucleus and Reticularis pontis oralis, the firing rate is higher in older rats during SWS but is similar in the wake cycle compared with the rate observed in younger rats. For young and old rats, the firing rate is higher during PS than the wake cycle or SWS, but the magnitude of this difference is higher in older animals. The variations of firing patterns observed during the wake–sleep cycle are also influenced by aging. In young and old animals, frequency of bursting is higher in PS compared with the wake cycle, but this difference is more important in older animals *(47)*.

Ultradian Cycle and Seasons

Several factors linked to neuronal activity are related to rhythmic activity, with periodicity differing from the circadian cycle. For example, ultradian fluctuations of neurotransmitter levels have been described for DA and noradrenaline (NA) for the whole brain *(48)*. This is also true in specific areas such as the hypothalamus, where release of DA and NA follows a cycle of 92 min and release of adrenaline occurs on a cycle of 99 min. A second oscillation with a 12-h period was also found for NA and adrenaline, and a 6-h period was found for DA *(49)*. These ultradian fluctuations were also described for 5-HT in striatum as well as for DA in cortex, striatum, septum, hypothalamus, and amygdala *(50,51)*. The number of receptors also fluctuates with an ultradian periodicity. In hypothalamus and the forebrain, α- and β-adrenergic receptor numbers reach a maximum level twice each day; these variations are specific to each receptor and follow an endogenous rhythm *(52)*. An ultradian variation of DA receptor is observed in striatum, with peaks at 2H00 and 14H00 *(53)*. These combined variations of neurotransmitter release and number of receptors probably are involved in the ultradian fluctuation observed for neuronal activity or drug effects *(54–56)*.

Factors that influence neuronal activity can also fluctuate through the year. The ultradian cycle of DA receptors in striatum has been shown to vary in amplitude waveform and time of peaks with the period of the year at which the experiment is performed. These variations are independent of the seasonal differences in the duration of the light period *(57)*. The same variation in ultradian cycle within the year was found for α- and β-adrenergic receptors peaks *(52)*.

Age

The age of animals used in experimental work has an impact on the outcome of the study independently of its interaction with the wake–sleep cycle. The impact of aging on neuronal activity is not homogenous and cannot be reduced as a global deterioration *(58,59)*. Some of the factors modified by aging that directly or indirectly affect neuronal activity are emphasized in this section.

Several morphological characteristics of the dendritic tree evolve with age. For example, in the cortex, the density of synapses between spine and axons peaks at 1 mo and then decreases with aging. The density of synaptic vesicles follows the same temporal evolution until it stabilizes in adult animals *(60)*. In hippocampus, the number, density, and surface of synaptic junctions change with aging. The number of synapses increases in youth and then decreases in older animals, whereas the synaptic surface does not differ between younger and older animals but decreases later in older animals *(61)*. Conversely, the size of the vesicles releasing neurotransmitters appears to increase with age, thus counterbalancing the loss in efficacy of synaptic transmission *(62)*. Receptors' affinity or their density can also evolve in an age-related manner; these changes are region-specific *(63–67)*. For example, *N*-methyl-D-aspartate (NMDA) receptors declined in CA1, CA3, and the subicullar region of the hippocampus but not in fascia dentate and the hilar region. Conversely, the number of adenosine monophosphate acid receptors decreased in fascia dentate and the hilar region but not in CA fields *(65)*. In the same brain area, the ratio between different subunits that form a specific receptor can change with aging. The magnitude of such effects across different regions is also different *(67)*. Altogether, these various effects contribute to a pattern of receptors—and therefore a pattern of activity—that evolve with aging.

In addition to these changes in receptor distribution, modifications of neurotransmitter effects have been observed with aging. Response to GLU agonist NMDA decreases in striatum and cortex *(68,69)* and the modulation of this response by DA in striatum are reduced *(68)*. These alterations of GLU responses are not limited to these two structures and can also be observed in non-NMDA receptors *(64)*. The reactivity to ACh and 5-HT also decreases with aging, despite unaffected membrane-passive properties *(70,71)*. Conversely, the reactivity to GABA appears to be preserved *(70)*. The extracellular level of neurotransmitters changes in different ways depending on the area studied. The level of DA increases in the somatomotor cortex but decreases in temporal cortex and hippocampus. For 5-HT, an increase is observed in both striatum and hippocampus, and norepinephrine levels increase in all cortical areas *(72,73)*. These changes underline the wide variety of aging-induced effects and ascertain that the process of aging cannot be viewed as a homogenous deterioration of brain function. These factors probably are also involved in the age-dependent variation of synaptic plasticity *(74–76)*. Together, these different age-dependent factors may contribute to modification of the electrophysiological properties and activity of neurons.

Stress and Enriched Environment

Some conditions may influence neuronal activity if they occur before (i.e., environmental enrichment) or during the experiment (i.e., stress). (For a review on enriched environment, *see* ref. *77*.) Interactions with the environment, and therefore the richness of stimulation from the environment, can have an impact on the morphology of neurons and can modify the number and length of dendrites and the number of spines held *(78–81)*. The affinity of receptors *(82)* or their number *(83)* are also sensitive to housing conditions and increase when the environment is enriched. These different factors probably enhance the efficacy of synaptic transmission between neurons *(84)* or, more broadly, the greater accuracy of response to sensory stimuli *(85,86)*. The stimulation induced by a novel environment is also sufficient to modify synaptic plasticity processes *(87)*. Within certain limits, the effect of enriched environment on neuronal activity can counterbalance, age-related alteration in the sensory system *(88)*. Environmental enrichment also limits senescence-related alteration of the wake–sleep cycle and increases the time of SWS and desynchronized sleep *(41)*.

During the experiment, the level of stress can also influence neuronal activity. For example, the level of DA increases when animals are

stressed, and this effect increases with daily exposure *(89)*. The levels of 5-HT in locus coeruleus and 5-HT and NA in locus coeruleus and amygdala are also influenced by stress *(90,91)*. For the latter, the variation in 5-HT and NA levels are observed only at the first exposure to the stressor. The stress level of the animal during the experiment can greatly influence the effect of applied stimulation *(92)*, and a stressor applied early in the life of the animal can also influence the activity of the brain *(93)*.

Diet and Metabolism

The well-being of laboratory animals is insured by housing conditions in a quiet and temperature-controlled environment with unlimited access to food and water. In certain experiments, however, animals can be fed for only a limited period during the day or with a limited amount of food. Dietary restrictions can have multiple influences on neurons *(94)*, neurogenesis *(95)*, and the level of neurotrophic factors in different areas of the brain *(96)*. The levels of neurotransmitters, such as serotonin or catecholamine, are influenced by the diet and show temporal variation during the feeding cycle *(97)*. The composition of the diet offered to animals can also modify drug-induced effects, such as haloperidol-induced changes in the activity of midbrain DA neurons *(98)*.

Sex and Hemispheric Asymmetry

Differences between the two cortical hemispheres can be found in neuronal activity related to sensory or motor systems *(99)*. Such differences are preserved in anesthetized rats, in which the responses evoked in prefrontal cortex are different between the right and left hemispheres *(100)*. In slice preparation, neuronal activity measured in the right or left suprachiasmatic nucleus in vitro are different *(101)*. Asymmetry has also been found in the dentate gyrus, and an enhancement of this difference has been evoked by seizure *(102)*.

The gender of the animals used has an impact on the results obtained. In 2000, Walker et al. *(103)* showed that release and uptake of DA in striatum were greater in female rats compared to males. These differences do not vary across the female estrous cycle *(103)*. Males have more muscarinic receptors in striatum and parietal cortex than females as well as a lower acetylcholinesterase activity in the parietal cortex and temporal cortex *(104,105)*. Finally, drug metabolism is different between males and females; for example, the half-life of phencyclidine is longer in the female rat brain *(106)*.

Summary

Many factors influence neuronal activity in freely moving or head-restrained animals. Differences observed between experiments may be linked to hemispheric asymmetry, gender, or diet of the animals used. Consider also that the environment to which the animal has been exposed before the experiment can influence neuronal activity at the single-cell level. Finally, biological rhythms of different periodicity that naturally occur in physiological conditions are responsible for important variations in basal activity as well as evoked response. These factors make it difficult to compare data from different studies with even slightly different experimental conditions.

Anesthetized Preparations

Background

Anesthesia can be used transiently to prepare animals for experiments as well as acutely to conduct studies requiring invasive approaches that are inappropriate with awake animals. Anesthesia maintains animals in a stable and immobile state, allowing experiments with cell-scale accuracy, which makes it popular for neuronal studies in vivo. Therefore, understanding the anesthetic agents' actions and their impact on the results obtained is essential when extending this work to physiological condi-

tions. Meyer *(107)* and Overton *(108)* proposed a molecular mechanism common to anesthetics that assume their action within the lipid bilayer. This unified theory offered the advantage of a non-agent-specific effect of anesthetic agents, which allowed easier interpretation of results obtained under anesthesia. The global activity of the brain was supposedly depressed by the disturbing action of the agent at the membrane level; therefore, regardless of the phenomenon studied, the magnitude of the effects observed were probably minimized but of the same nature of what should have been observed without anesthesia. With accumulating evidence of proteins as targets for agent-specific effects, this theory has been challenged and alternative hypotheses have been formulated *(109,110)*. Some of these recent developments, their implications, and the still widely unknown impact of anesthesia on neural cell physiology are presented in the following sections.

Anesthetics Modify Membrane Excitability

Two-Pore Domaine Potassium Channels

Neuronal excitability can be modified directly by anesthetic action on background potassium channels (also known as two-pore domain potassium channels; ref. *111*). This effect of anesthesia is independent of neurotransmitter-sensitive current as well as the membrane voltage. These channels are the targets of various anesthetic agents, such as nitrous oxide, xenon, cyclopropane *(112)*, and volatile anesthetics *(113,114)*. These agents enhance the hyperpolarizing action of these channels. Two of these potassium channels (TASK and TREK-1), which are present in the brain and share similar biophysical properties with inhibitory synaptic current, are specifically activated by volatile anesthetics. TREK-1 is activated by chloroform, ether, halothane, and isoflurane, whereas TASK is activated by halothane and isoflurane *(115–118)*.

Recent studies have described some radically different modulators of these channels. The endocannabinoid anandamide can specifically block the two-pore domain potassium channel independently of an action on the CB1 and CB2 receptors. This suggests that interaction may exist between the endocannabinoid pathway and the mechanism of action of volatile anesthetics *(119)*. A modulation by 5-HT has also been proposed *(120)*. By their global action on neuronal excitability, these receptors play a major role on anesthetic-mediated changes of electrical properties of neurons. Further investigation regarding their modulation and anatomical location will enhance the understanding of volatile anesthesia-induced alteration of neurophysiological data.

Anesthetics Can Affect Voltage-Gated Receptors

Voltage-gated receptors are also the targets of anesthetics agents and, therefore, deeply modify the electrical properties of the neuronal membrane. For example, halothane and enflurane can reduce the current induced by the voltage-dependent calcium (Ca) channels *(121)*. These agents do not modify the basal level of intracellular Ca but limit its increase that is induced by stimulation *(122)*. The Ca conductances are also involved in the burst firing pattern that can be blocked by isoflurane *(123)*. Not all voltage-gated Ca channels are sensitive to anesthetics. The so-called "P-type channel," which constitutes the largest population of these channels in the mammalian nervous system *(124)*, is unaffected by a wide variety of anesthetics (e.g., halothane, isoflurane, thiopental, ethanol, pentobarbital, propofol; ref. *125*). Ketamine and propofol inhibit voltage-sensitive potassium current *(126)*, whereas isoflurane increases it *(127)*. If this opposite effect can be linked to the different anesthetics tested, it is important to emphasize that the models used in these studies are critically different; this point may be paramount, as we attempt to demonstrate in this article.

The effects of anesthetic agents on voltage-sensitive channels discussed earlier are involved in the alteration of the generation of spikes. In fact, halothane, isoflurane, and enflurane can change the spontaneous firing pattern of CA3 neurons from solitary spikes to burst activity

but do not affect the resting membrane potential *(128)*.

The voltage-dependent channels involved in the conduction of electrical impulses in nerve fibers are also influenced by anesthetics. For example, isoflurane can decrease conduction velocity of nerve fibers, and more importantly, its effects on myelinated and unmyelinated axons are different *(129)*. On the other hand, the volatile anesthetic halothane has no effect on conduction velocity in thin, lightly myelinated fibers but does depress amplitude of the action potential volleys *(130)*.

Anesthetics Modify Neurotransmitter-Induced Effects

Beyond their direct action on membrane electrical properties, anesthetic agents appear to be very potent ligands to neurotransmitters receptors. Anesthetic agents likely principally exert their action through the modulation of these receptors' activities *(131–133)*. The effects of different anesthetics, principally on GABA and GLU receptors (GLURs), are presented in the following section.

GABA Receptors

GABA is the major inhibitory neurotransmitter in the central nervous system. Numerous anesthetics can bind GABA-ergic receptors, and the modulation of this receptor was proposed as a major component of the anesthetic effects of this agent. The GABA receptor associated with the Chloride channel is predominantly involved in this mechanism *(134)*. Nevertheless, ketamine appears to be an exception and has only a minor—if any—effect on GABA receptors *(135,136)*.

A common mechanism seems to explain the enhanced GABA-induced inhibition by intravenous and volatile anesthetics. In fact, the GABA-induced current is enhanced by propofol, trichloroethanol, and volatile anesthetics *(137–146)*. The chloride conductance through this channel can be directly modulated by the anesthetic or mediated by intracellular Ca *(147)*. Interactions between GABA and its receptors are also modified by anesthetics. For example, sevoflurane can increase the affinity of the receptor or block it in an open position *(143)*. The effects described earlier depend on the subunit composing the receptors *(148,149)*. Together, these mechanisms are responsible for the enhanced inhibitory action of GABA under anesthesia *(150)*. The pre- or postsynaptic location of the GABA receptor targeted by the anesthetic agent is also important for its overall effect, and each site is affected by a specific anesthetic to a different extent *(146)*.

Studies regarding the impact of anesthesia on electrical activity of neurons have shown that the spontaneous firing rate, as well as the firing pattern, is affected in an agent-specific manner. Patterns of activity observed in the normal wake–sleep cycle are enhanced by anesthetics, and the magnitude of these changes depends on the agent used *(151,152)*.

Glutamate Receptors

Volatile anesthetics exert an inhibitory effect on glutamatergic transmission in different areas of the brain *(140,145)*. They depress GLU-mediated excitation through a mechanism involving pre- and postsynaptic mechanisms *(137,153,154)*. Some of this effect depends on the type of GLURs present. NMDA receptors are more sensitive to certain agents (such as isoflurane) than non-NMDA receptors. This selective effect has not been observed for halothane *(137,153)*. The importance of the subunits composing the receptors recently has been emphasized. Opposite responses to kainate under volatile anesthetics were observed, depending on the subunits that composed the tested receptor *(155)*. Sensitivity to anesthetics also varies with the age of the animal, probably because of the attenuation of synaptic excitation observed with aging *(156)*. Finally, an alteration of the GLU neurotransmission by anesthetics has also been proposed to increase bursting activity *(157,158)*.

Some intravenous anesthetics such as trichloroethanol, the active metabolite of chloral hydrate, also have an inhibitory effect on glutamatergic transmission *(159)*. This agent

affects GLURs differently, with increasing sensitivity from NMDA to quisqualate to kainate. Urethane inhibits NMDA- and non-NMDA-induced current *(160)*, whereas pentobarbital inhibits only the non-NMDA-induced current *(161)*. For the latter, the inhibitory effect on kainate response depends on specific subunits that compose the receptors *(155)*. Volatile and intravenous anesthetics can alter GLU-induced activity differently, depending on the type of receptor present.

ACh, Glycine, and Dopamine Receptors

ACh receptors are also sensitive to some anesthetic agents—more specifically, the nicotinic subclass of receptor is modulated by volatile and intravenous anesthetics *(162,163)*. The ACh-induced current through nicotinic receptors is inhibited by ketamine in a reversible and dose-dependent manner *(164,165)*. Cholinergic agonists tested on neurons from the somatosensory cortex had a weaker effect when tested under urethane anesthesia *(47)*, whereas ACh had less effect in the medial septal area under anesthetized conditions. This effect appears specific to ACh-sensitive cells in this area because no differences were observed in the number of GLU-activated or GABA-inhibited neurons between anesthetized and urethane anesthetized animals *(166)*. Glycine-sensitive receptors are also targeted by anesthetic agents *(140)*, and volatile anesthetics can enhance the glycine-mediated current using different mechanisms *(167)*. A modulatory action of the D_2 dopaminergic receptor by volatile anesthetics has also been proposed *(168)*.

Effects of Anesthetics on Neurotransmitters Release and Uptake

Neuronal activity is also altered by anesthesia through its impact on neurotransmitter release and uptake. A few examples of the alterations induced on some neurotransmission systems and their underlying mechanisms are provided here. Ketamine enhanced the electrically evoked release of DA in striatum and slowed its re-uptake *(169)*. The opposite effect on evoked release is observed under halothane anesthesia, probably because of modulation of the D_2 receptors *(168)*. Halothane and isoflurane also induce a decrease of DA release that is chemically evoked by NMDA. This effect results from the combined presynaptic depression of NMDA response and an enhanced GABA transmission *(170)*. This increased GABA transmission, via enhanced release, was also observed with in vitro cortical preparation *(171)*, whereas GLU levels were decreased by an enhanced uptake *(172)*. With ketamine, the extracellular levels of GLU increase in medial prefrontal cortex; this effect is mediated by Group 2 metabotropic GLUR *(173)*. Therefore, anesthetics can affect various neurotransmission systems in opposite ways by acting on release or uptake systems.

Anesthetics can produce identical effects, despite opposite action mechanisms. Ketamine increases 5-HT in Nucleus Accumbes for a prolonged time, and the same—although shorter—effect is observed with chloral hydrate. These two anesthetics, as well as pentobarbital, suppress the morphine-induced increase of 5-HT, but the pentobarbital decreases the extracellular level of 5-HT *(174)*. Despite an opposite effect on 5-HT levels, these anesthetics can suppress the drug-induced increase of this neurotransmitter. Serotonin-evoked efflux in dorsal raphe nucleus is also enhanced by ketamine, whereas re-uptake is slowed; the same effect was observed in the bed nucleus for NA. The amplitude of both effects was different for each anesthetic *(169)*. Most neurotransmitter release and re-uptake systems appear to be affected by anesthesia. Apparently, accounting for an anesthetic effect on these systems is not sufficient to anticipate the alteration of drug-induced effects from anesthesia.

Sensory Stimulation and Pain Under Anesthesia

In acute experiments realized under anesthesia, it is assumed that the brain is isolated from the outside world and that the steady state observed is free of artifacts induced by

changes in the environment. However, even if the reactions to sensory stimulation are deeply affected by anesthesia, they are not totally suppressed. Under isoflurane, somatosensory-evoked response in thalamus is decreased but not blocked *(175)*. The same effect has been observed in the cortex under urethane, ketamine, and pentobarbital, despite the very different properties and action of these anesthetics on neurotransmission *(176,177)*. The evoked responses of the cells are not only modified by anesthesia, but their basal activity is affected as well *(178)*. Anesthesia can specifically modify responses to stimulation of one body area as well as responses of specific subpopulations *(179)*. Some cells can be strongly activated, or the number of cells from one subclass can increase *(180,181)*. Finally, an enhanced response to innocuous stimuli can be observed under pentobarbital anesthesia *(182)*. This enhanced inhibitory response to sensory stimulation is consistent with the molecular mechanism of pentobarbital *(183)*. Sensitivity of other sensory systems is altered by anesthetics; for example, responses to auditory stimuli are depressed but not eliminated *(184)*. Anesthesia decreases but does not block the cortical response to sensory stimuli. It is particularly important to note that such stimulation induces a different kind of neuronal activation under anesthesia than in anesthetic-free conditions.

In the specific context of an acute experiment, the blockade of noxious stimuli by anesthetics is particularly important to obtain results free of artifacts linked to the subsequent pain endured in experimental conditions. The absence of observable reactions to the surgical procedure is not sufficient to conclude on the depth of anesthesia. Anesthetic agents used at concentrations that inhibit motor responses to noxious stimulation do not suppress the variations in blood pressure and heart rate that are induced by these stimulations *(185)*. As described earlier, sensory stimulation anesthesia modifies neuronal response to noxious stimuli and does not block them *(179,182)*. Some of these effects are agent-specific, with more neurons inhibited by nociceptive stimuli with pentobarbital than by urethane and ketamine anesthesia *(186)*. Interestingly, anesthesia-induced changes of the neurophysiological functions are so radical that subpopulations of noxious-sensitive cells exist only under anesthesia *(181)*. From this short review of the literature regarding sensory and noxious stimuli effects on neuronal activity under anesthesia, we can conclude that under these conditions and with every agent tested, the brain is still influenced by the environment. Therefore, tactile stimulation or pain can elicit activation and inhibition that are likely to induce artifacts.

Temperature and Anesthesia

Anesthesia can indirectly alter neuronal activity by its impact on thermoregulation. Under anesthesia, mammals lose their ability to thermoregulate, and thermal blankets are often used to compensate. The body core temperature is used as a reference point to warm the animal to ensure a stable condition close to the physiological one. For example, chloral hydrate or α-chloralose anesthesia can make the brain temperature drop by 2.5 to 4ºC below the body temperature, which is maintained at 37.5ºC with a heat exchange platform *(187)*. In one experiment, the temperature gradient observed in the brain was also different between the two anesthetics, but deeper structures were always warmer. Anesthesia can impair thermoregulation; however, in return, the action of some anesthetic agents is also sensitive to temperature. In fact, the inhibitory effect of volatile anesthetics on nicotinic ACh receptors increases with lower temperature *(188)*. The same effect has been observed on GABA A receptor, and the potentiation observed was agent-specific only at low temperatures *(189)*.

It appears that despite a normal body temperature, anesthesia can lower brain temperature and that brain temperature is not homogenous *(190)*. This factor is probably of major importance regarding anesthesia studies for interpretation in a behavioral context. The influence of temperature on neurophysiology has long been acknowledged *(191)*. Under anesthesia,

the latency of evoked response in the cortex varies more as a result of the body temperature than of the anesthesia *(192)*. The amplitude of the different phases of these responses is affected differently when the cortical temperature varies locally. The background activity is also affected by body or local temperature, but this occurs in an opposite manner *(193)*. When studied at the single-unit level, temperature shows an influence on the shape of individual spikes and the spontaneous firing rate. An increase in temperature reduces both the height and width of action potential, whereas it increases the spontaneous activity and reduces response to stimulation *(194)*.

Small variations of temperature (1–1.5°C) also occur in behaving animals, and despite their limited amplitude, they are responsible for some variation observed in neuronal activity. More specifically, field potential parameters can increase under the influence of passive warming, and there can be an increase in temperature linked to muscular activity *(195,196)*.

Summary

To summarize, when results obtained with anesthetized animals must be interpreted in the context of freely moving animals, several parameters must be considered. All anesthetics have an effect on the excitability and neurotransmission of neurons, and as discussed previously, such effects can be different between anesthetic agents or neuron populations. The importance of metabolites released from the degradation of anesthetic agents is also a consideration. Chloral hydrate, a widely used anesthetic agent in experimental research, has only a weak intrinsic anesthetic effect. Most of this effect comes from one of its metabolites, trichloroethanol. Considering this level of interaction makes it difficult to evaluate the bias induced by anesthesia on spontaneous or induced neuronal activity. To avoid this problem, it might be interesting to compare results from the same experiment obtained under different anesthetics. The reciprocal action of anesthetics on temperature is also important, and a stable brain temperature close to the physiological temperature is crucial. Finally, even under anesthesia, the brain cannot be considered as isolated from changes in the environment. Noxious and innocuous stimuli can reach several areas of the brain and, consequently, can induce some variability in observed activity. This emphasizes the need for controlled and stable environmental conditions, even in experiments performed under anesthesia.

In Vitro Slice Preparation

Background

The in vitro slice preparation provides a unique opportunity to study neurophysiology in well-controlled conditions with direct access to neurons *(197,198)*. Many methodological aspects are critically important regarding the usability of the brain slice and the accuracy of the results obtained *(199)*. Important criteria regarding slice health are still studied and improved to optimize the in vitro approach and its representation of in vivo conditions *(200)*. This article reviews some of the essential factors that are useful when interpreting experimental results from behaving animals based on work obtained in slice preparations.

Metabolism

The injury resulting from brain slicing is a combination of ischemia prior to slicing and trauma induced by cutting. Such damage is particularly important on the edges of the slice *(201)*. Incubation parameters can be optimized to ensure cell survival and neuronal activity in the range of in vivo parameters for several hours *(202)*. The thickness of the slice is critically important and influences the survival of neurons, the preservation of their morphological integrity, their mean firing rate, and the effects of temperature *(203,204)*. The overall survival of neurons in a slice depends on the age of the rat used to prepare it; slices from

adult animals show more damage. It is particularly interesting to note that deterioration of cell populations is not uniform *(205)*. This different vulnerability to the in vitro condition also depends on time, with specific subclasses of cells degrading earlier than others *(206)*.

Oxygen Concentration

Oxygen is critically important for the survival of neurons, and a shortfall in the supply of such a vital element during the preparation or the experiment can be expected to influence the results obtained. When slices are isolated from the rest of the brain, the duration of the resulting transitory ischemia can modify evoked neuronal activity *(207–209)*. It may take up to 1 h to recover maximal evoked electrical response. In classical working conditions, the slice is bathed with a medium bubbled with a mixture of 95% O_2 and 5% CO_2. This oxygenation procedure ensures a high concentration of dissolved oxygen and, therefore, its availability for neurons. Nevertheless, the O_2 concentration decreases in the depth of the slice, and this decrease is linked to its diffusion in the slice and its consumption by the cells *(210)*. In the center of thick slices, the O_2 concentration can be close to null. Additionally, evoked activity can increase the O_2 consumption with an important delay and in a dose-dependent manner. This increased O_2 consumption, coupled with low O_2 availability, can create locally hypoxic conditions responsible for the decreased amplitude of postsynaptic response *(211,212)*.

This locally induced hypoxia can limit synaptic transmission by various mechanisms such as membrane hyperpolarization, a decrease in postsynaptic potential, and increased membrane conductance. However, neurons can recover a normal evoked response after a period of hypoxia as long as 20 min; for that period, the morphology of the neurons is indistinguishable from the nonhypoxic control condition. No recovery occurs when the slice is exposed to 60 min of hypoxic condition, and in that case, swollen neurons outnumber intact cells *(213)*. Zimmerman et al. *(214)* provided an illustration of differences in O_2 supply between in vitro and in vivo preparations. In anesthetized animals, an electrical stimulation of the medial forebrain bundle increases O_2 concentration in caudate nucleus, whereas in slice preparation, the electrical stimulation results in a prolonged decrease of O_2 concentration. The decrease observed in vitro results from an increased O_2 consumption that is not compensated by oxygen diffusion within the slice. In intact animals, the peak of O_2 concentration observed probably results from the increased blood flow mediated by ACh *(214)*.

Neuronal activity is extremely sensitive to oxygen availability that is not homogenous in a slice. This gradient from surface to center can be locally modified by cell metabolism, creating locally hypoxic conditions. Overall, these variations of O_2 availability may modify the activity of neurons integrated in the same network, even if their connections are preserved in the slice.

Receptor Expression and Protein Synthesis

Neuronal activity is partly conditioned by neurotransmitters' actions on their receptors present at the membrane. Changes in the number, activity, or affinity of these receptors can have a major impact on the electrical activity of neurons. The distribution pattern of receptors is also critical when studying neurotransmission at the neuronal level. This pattern is sensitive to the slicing procedure used for in vitro slice preparation. Focusing on adenosine monophosphate acid receptors, Taubenfeld et al. *(215)* showed that specific subunits of this receptor disappear quickly after the preparation of the slice, whereas the concentration of other subunits remains stable. Beyond the loss of some specific proteins, the synthesis *de novo*, which is sometimes proposed to explain long-term changes, must also be considered carefully when discussing experiments obtained in different models. When studied in vivo, this synthesis appears homogenous in adult animals, whereas regional differences exist in younger animals. It is still unknown whether such differences are conserved in other preparations *(216,217)*. Overall, when the synthesis

rate is studied in vitro, striking differences appear between slices obtained from young and adult animals. Slices from young rodents retain 80% of the in vivo synthesis rate, whereas this rate falls to 10 to 20% in slices from adults *(216–220)*. This metabolism depends on temperature, with a rate twice as high at 35°C compared with 30°C and a fivefold increase between 25 and 35°C *(218)*.

Temperature

In slice preparations, a temperature lower than the physiological temperature is often used to improve the viability of neurons. This must be considered carefully in the interpretation of the results obtained, because the properties of neuronal membranes and generation of action potential are dramatically influenced by temperature. Cooling by a few degrees is enough to depolarize neurons, increase their membrane resistance, and increase spike amplitude in areas such as cortex or hippocampus *(10,11,221)*. The conduction velocity of intracortical fibers is also dramatically affected by temperature, with an increase of 60% (a Q10 of 1.6) between 25 and 35°C *(154)*; cooling also alters the synaptic transmission. A decrease of temperature lowers the probability of release and slows the replenishment of the releasable pool of neurotransmitters *(9,10,222)*. Evoked and spontaneous release of neurotransmitters strongly depend on the temperature *(223)*.

The presence of neurotransmitters in the synaptic cleft, and therefore synaptic transmission, is also regulated by the uptake of neurotransmitters. Al-Hayani and Davies*(224)* provided an illustration of the radical effect of temperature on this mechanism. These authors showed that the effects of cannabinoids on synaptic transmission at 28 to 30°C are opposite of those obtained at 35°C. An increase of GABA uptake with temperature appears to be responsible for this difference. The same reuptake mechanism also limits the diffusion of neurotransmitters in the extrasynaptic space and minimizes crosstalk between synapse at physiological temperature *(225)*.

Together, these modifications of the electrical properties of neurons are involved in the longlasting potentiation of population spikes (summed discharge of pyramidal cells), the modified effect of repetitive stimulations *(226,227)*, and the different evoked responses observed with temperature changes *(154,193, 221,228)*.

Morphology

In addition to the effect of temperature on the electrical properties of neurons, recent studies have uncovered morphological changes at the dendritic level that can deeply modify the activity of neurons *(12,229)*. The preparation of the slice may increase the number of spines by 40 to 50% in slices obtained from adult rats compared to that observed in perfusion-fixed tissue. This effect depends on the age of the rat; in the same group of postnatal 21-d-old rats, the spine density increase can reach 90%. These dendritic spines have normal synapses with both pre- and postsynaptic elements and, therefore, are probably fully functional. Different categories of spines are not affected equally between adult and immature slices, and these modifications appear within 2 h and are stable for at least 13 h *(12)*.

The transitory incubation at low temperatures often used in the preparation of slices *(230)* causes dendritic spines at first to disappear and then to proliferate following rewarming to the recording temperature. The newly formed spines are longer and more numerous than those observed in a preparation representative of in vivo morphology *(229)*. A moderate cooling period of 3 h is enough to stop actin-based motility of spine heads. Spines that do not exist or only rarely appear in vivo can also be observed after slicing. Their number increases in a time-dependent manner *(231)*. Synaptogenesis is sensitive to the condition of slice preparation as well as neurotransmitter release, glycogen loss, and microtubule assembly *(232)*.

Synaptic connectivity is a key factor regulating the electrical activity in neuronal networks. Their structures appear to be mostly quantitatively sensitive to the procedure of slice prepa-

ration. This may enhance the responsiveness of some specific pathways to stimulation, and this facilitates their study but also undermines interpretation at a more integrated level.

Network Activity

Rhythmic activities of the assembly of neurons naturally exist throughout the brain in physiological conditions (*see* Circadian Cycle and Ultradian Cycle and Seasons sections); the periodicities of these cycles are in the range of minutes to hours. When slices are isolated from the rest of the brain, rhythmic activities are conserved if the slice holds the neurons that generate the pacemaker-like activity *(233)*. This provides evidence that in vitro slice preparation can retain patterns of activity observed in physiological conditions.

To minimize the impact of several factors presented that influence neuronal activity in slice preparation, small populations of neurons can be isolated *in situ* from the rest of the brain *(234)*. This preparation retains the local organization between cells and rhythmic activity generated by specific neuronal populations within the slab; after axotomy, the rhythmic activity disappears outside of the slab *(235)*. Eliminating these rhythmic activities provides the opportunity to study neuronal activity in stable conditions that are lacking in behaving animals and that limit interpretations of results as discussed earlier.

The spontaneous oscillating activity observed in intact cortex can also totally disappear when small slabs are isolated. With larger territories, the oscillation is partially preserved, and their properties converge toward the one observed in intact cortex *(234,236)*. Preserving this rhythmic activity is particularly important because they are relevant to the wake–sleep cycle, neuronal plasticity, and seizures (for further discussion on network activities and their importance, *see* ref. *8*).

Summary

To summarize, different factors must be considered before using results obtained with in vitro slice preparation to interpret experiments realized with other experimental models. The incubation parameters of the slice, such as temperature, should be as close as possible to the physiological parameters, because these can modify electrophysiological results. As discussed previously, slices isolated from young brain survive better in vitro and, therefore, are preferred. Nevertheless, the differences observed in neuronal activity at various ages plead for age-matched animals between the two sets of compared data.

Within the slice, the structural integrity of neuronal connections is preserved but oxygen diffusion, temperature-dependent morphological re-organization, or protein turnover may limit the interaction between cells compared to in vivo situations. Finally, the isolation of a small group of neurons can be considered an advantage because in this condition, it is possible to study neuronal activity free of input from cells outside of the slice. Conversely, the isolation of a slice from the rest of the brain greatly limits the emergence of specific patterns of activities that are relevant to physiological conditions.

Conclusion

To fully benefit from the large body of work that is produced in neurobiological research, experimental results from different models must be assembled in a coherent manner. As discussed earlier, neuronal electric activity can be influenced by many factors that depend on the experimental conditions or can fluctuate spontaneously. A remote difference in a parameter, the timing of an experiment, or the natural history of the animal used in an experimental study can induce large differences in the results obtained. Together, this makes it difficult to compare experimental data with different origins.

The impact of a fundamental parameter, such as temperature, on the outcome of an experiment is probably the best example of the difficulties that arise when combining or com-

paring results from different experiments. Temperature fluctuation before or during experiments may influence different factors at different times. Cooling neural tissue before an experiment can modify the dendritic spines and, therefore, the excitability of neurons. During the experiment, changes in temperature influence the properties of neuronal membranes, the conduction of nerve fibers, and neurotransmitter uptake and release. Temperature also greatly influences the natural turnover of neurotransmitter receptors or their induced synthesis. However, in vitro preparations are not the only temperature-sensitive models. When working with anesthetized animals, it is usually assumed that a physiological body temperature is enough to obtain a normal brain temperature. However, different studies have challenged this assumption and have emphasized that this divergence from physiological conditions is likely to have an impact on neuronal activity *(187,190)*. Conversely, temperature does not appear to be steady in freely moving animals, and it fluctuates, within narrow limits; these factors are probably impossible to model in vitro *(237–239)*. This raises the issue of which models should be used as a reference; indeed, if freely behaving animals appear to best represent physiological conditions, then this experimental model does not provide the stability required in most experimental procedures and thus greatly reduces the research that can be performed with such a model.

The extensive literature describing neurophysiological studies and their results does not—and will probably never—fully embrace the complexity of neuronal activity in each of the models used for experimental research. Neuronal activity is driven by many factors, some of which are well-identified in various conditions (i.e., physiological or not) and have been described previously. By using the available information, it is probably possible to overcome some of the limitations imposed in data interpretation by technical parameters or limited/simplified models.

Ultimately, studies involving different experimental models or a set of experimental conditions within the same model could help build universal knowledge. When coherent experimental results are obtained within the same study but using different techniques (both in vitro and in vivo), different conditions, and different animal strains, the conclusions drawn are greatly strengthened and more probably universal *(240)*.

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