NEUROPHYSIOLOGICAL METHODS IN ANAESTHESIA RESEARCH*

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STUDIES by Larrabee and Posternak¹ on sympathetic ganglia demonstrated that some anaesthetics depress synaptic transmission more readily than the process by which the action potentials are propagated. Recent experiments on the spinal cord monosynaptic reflex have confirmed these observations.²⁻⁵ However, the mechanism responsible for this depression is not known. The synaptic process is complicated and anaesthetics might act at different sites,⁶ for instance, at the afferent terminals, at the synaptic gap, or at the post-synaptic membrane.

Furthermore, synapses can be classified as excitatory and inhibitory. The latter type regulates the input and output of specialized pathways in the form of pre- and post-synaptic inhibition.

Studies of the effect of anaesthetics on synaptic transmission are frequently difficult to interpret because: (a) the synapse is not located in the central nervous system (CNS) or (b) most experiments are performed on anaesthetized animals or (c) the administration of the anaesthetic, *per se*, alters the animal's systemic conditions.

This paper describes some methods of studying the effect of anaesthetics on synaptic transmission in the CNS of unanaesthetized cats. A summary of the action of local and systemic administrations of procaine, pentobarbitone, and halothane is presented.

METHOD

Synaptic transmission was studied in the cuneate nucleus. This nucleus represents the first synaptic relay of the main sensory pathway originating in the forelimb and terminating in the cerebral cortex. This pathway is also known as the dorsal column-medial lemniscal system. Most of the axons of cuneate relay cells form part of the medial lemniscus that ends in the ventroposterolateral nucleus of the thalamus. Other axons and/or collaterals terminate in the cerebellum.

The cuneate nucleus is located in the dorsum of the medulla; it has a rostro-caudal length of approximately 10 mm. and a dorsoventral thickness of 2 mm. at its maximum. The terminal fibres of the dorsal columns cover the dorsal surface of the nucleus before they bend, almost at a right angle, to terminate in the nucleus itself. The two cuneates are separated from each other by the more medial gracilis nuclei.

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SURGICAL PREPARATION

The experiments were performed on adult cats of either sex and with an approximate weight of 2.5 kg. Plastic catheters were placed in the femoral artery and femoral vein. The former was connected to a manometer. The latter was used for the administration of various drugs and solutions. A heating pad thermostatically regulated by a rectal probe was used to maintain a central temperature of $36^{\circ} \pm 2^{\circ} \text{C}$.

The muscles of the neck were separated. Part of the occipital bone and the posterior surface of the atlas were removed to expose the upper spinal cord and lower medulla. The dura and arachnoid were removed under a dissecting microscope. A small area of the pia over the left cuneate nucleus was removed for future penetration of micropipettes. Decerebration was performed using the following technique developed during the course of these experiments: anaesthesia was induced with a mixture of ethyl chloride and then of ether given through a face mask. Once the animal became unresponsive to painful stimuli a metallic cannula was passed through a tracheostomy and the administration of ether continued with the aid of a special adapter. Both common carotid arteries were tied, and the superficial muscles, the oesophagus, and the trachea were sectioned between ligatures. The rostral part of these tissues was pulled back; the structures ventral to the skull and the first vertebrae were exposed with the aid of a retractor.

The two muscles, longus capitis and rectus capitis anterior minor, were separated from the surface of the occipital bone at the base of the skull. A hole 5 mm. in diameter was drilled between the two bullae tympani. The dura was removed and a metallic clip placed on the basilar artery. The objective of this ligature was to minimize blood loss during decerebration. Under these conditions it is possible by direct vision to localize the medial lemniscus and the pyramidal tract at the point of section. Minimal bleeding helped to maintain most of the experimental preparations in good condition throughout the experiments.

The anaesthesia was discontinued after removal of the brain above the bony tentorium. The left superficial radial and the median nerves were dissected and mounted on bipolar silver wire electrodes specially adapted to a plastic cuff so as to make a "minipool" of mineral oil (Fig. 1).

Artificial respiration, by means of a "Palmer" pump, was used whenever paralyzing agents were administered. Oxygen at various concentrations was given as required. A bilateral thoracotomy was performed in most experiments to prevent the displacement of the medulla with respiratory movements and facilitate single unit recording.

The effect of anaesthetics on the afferent terminals was determined by the changes in size of the antidromic compound action potential recorded in the superficial radial nerve, stimulation being made from the cuneate nucleus. The effects on the post-synaptic membrane were assessed by the changes in the rate of firing of cuneothalamic neurones excited by the microiontophoretic release of L-glutamate. The effect on inhibition was evaluated by change in the trans-synaptic

FIGURE 1

depression induced by a conditioning pulse to a peripheral nerve on the action potential evoked by stimulation of a second nerve. Overall synaptic efficiency was determined by the size of the medial lemniscus (or cuneate nucleus) evoked action potentials.

Anaesthetics were administered using the following techniques: (a) microiontophoretic, (b) electro-osmotic, (c) pressure, (d) local perfusion and (e) systemic (intravenous—nembutal; inhalation—halothane).

The microiontophoretic method was first applied by del Castillo and Katz⁷ and Nastuk⁸ on the myoneural junction. Later Curtis⁹ and co-workers developed the multi-barrelled pipette for the simultaneous study of several compounds. This same electrode has been used in the spinal cord for stimulation, recording, and administration of procaine and other substances.

The use of a special cup for the surface perfusion of halothane dissolved in Ringer's solution (Fig. 2) facilitates the administration of this agent at known concentrations without the systemic interference produced by changes in respiration or blood pressure.

FIGURE 2

MICROIONTOPHORESIS

The microiontophoretic technique of drug administration is based on the release of ionized chemical substances from fine glass pipettes by an electrical current. This movement can be controlled by the magnitude of the potential gradient applied across the conducting solution inside the pipette. If the solution is made positive relative to the tissue, cations will be carried out of the tip as a cationic current. If the tip is made relatively negative, anions are then carried out by anionic current. In order to prevent spontaneous release of active ionic compounds, a small current of opposite polarity to that of the active ion is permanently applied to the electrode. This current is known as the "backing current."

The amount of a given ion released by iontophoresis can be calculated by the following equation:

$$M = nI/ZF \quad (1)$$

where M is the ionic flux (moles/sec.), n the transport number, I the current, F Faraday's constant, and Z the valency. Thus, the ionic flux is not directly known from the current flow since the exact value of n is not known. However, for a

given pipette the flux is usually proportional to the strength of the current;¹⁰ consequently it is reasonable to study the relative activity of a given drug at different current strengths.

Except in the immediate vicinity of the tip,¹¹ once the ions are liberated the potential gradient in the tissue is too insignificant to affect their distribution in the external environment. The concentration that can be obtained depends upon the rate of ejection and the characteristics of the extracellular (or intracellular) space. The concentration at a given distance is governed by diffusion laws. In an infinite homogenous medium, a steady efflux (M) from a point source should theoretically give the following distribution of concentration with time and with distance:

$$C = \frac{10^7 M}{4\pi x D} \times \frac{x \cdot 10^{-4}}{4Dt} \quad (2)$$

C is the concentration in moles/L. at a distance x from the tip at time t (in seconds) after the beginning of the ejection at a constant rate M moles/sec. D is the diffusion coefficient of the substance in the external medium. For long periods of time this equation can be reduced to:

$$C = \frac{10^7 M}{4\pi x D} \quad (3)$$

ELECTRO-OSMOSIS

The application of a potential gradient and the resultant current across a microelectrode not only releases ions by iontophoresis but induces an electrokinetic flow of solvent, which may also carry out inert or charged particles. This electro-osmotic ejection depends upon the concentration of the solution and its nature, as well as the type of glass used in the fabrication of the pipette.

$$V = \frac{\xi r^2 e E}{4\eta\pi(300)^2} \quad (4)$$

where V is the volume (in ml.) of liquid transported each second through a tube of length l (in cm.) and radius r (in cm.). E is the applied potential in volts and ξ is the zeta or electrokinetic potential (volts); e is the dielectric constant of the solvent, and η is the coefficient of viscosity of the solution. This equation can be simplified to:

$$V = \pi r^2 u E \quad (5)$$

where u is the electro-osmotic mobility (in cm./volt/sec.). For a micropipette, $V = \rho I u$, where I = current passed and ρ the specific resistivity of internal solution.

The contribution of electro-osmosis to the total amount of drug released when a potential gradient is applied to a pipette is negligible in concentrated solutions, as demonstrated by Krnjević *et al.*¹² by measurements with ACh. However, electro-osmosis can be used as the principal method of release when working with very low concentrations ($\text{NaCl} \leq 0.01 M$) and non-ionizing drugs such as the

inhalation anaesthetics. These solutions are positively charged with respect to Pyrex glass,¹³ and they are released by cationic currents. However, it is difficult to determine the actual amount being released, a circumstance that prevented us from using this technique, except for a few experiments made to assess its possibilities and limitations.

PRESSURE

Several investigators have used pressure to eject drugs from micropipettes.¹⁴⁻¹⁶ However, owing to the high resistance offered by electrodes of 1 or 2 μ diameter, mechanical displacements, and possible damage to the cell by the volume of solvent being injected, this technique was used by us only in a few experiments that confirmed these theoretical disadvantages.

Iontophoresis, electro-osmosis, and pressure were used in conjunction with the five-barrelled micropipette. The central barrel was filled with a 2.7 M solution of NaCl for recording (or stimulation in some experiments). The other four barrels were filled with solutions of various compounds. In most pipettes one of these barrels had a 1 M solution of NaCl which was used to determine current artifacts. Halothane was injected by pressure from a saturated solution of .01 M NaCl placed in the central barrel. A lateral barrel was then used for recording.

DRUGS

Na L-glutamate, 2 M, pH 6.5 (British Drug Houses)	Nembutal, .2 M, pH 9.5 (Abbott)
γ -aminobutyric acid, 1 M, pH 3-5 (California Biochemicals)	Procaine hydrochloride, 1 M, pH 5.4 (Abbott)
Glycine, 1 M, pH 4 (Fisher)	Veratrine, .2 M, pH 3.3 (British Drug Houses)
gallamine triethiodide, .5 M, pH 6.3 (Poulenc)	tubocurarine chloride, .02 M, pH 6.3 (Burroughs Wellcome)
strychnine sulphate, 0.2 M, pH 6.4 (British Drug Houses)	succinylcholine chloride, .5 M, pH 6.3 (Brickman and Company)
Picrotoxin, .02 M, pH 9.5 (British Drug Houses)	halothane B.P. (Hoechst)

The pH of these substances was adjusted with .1 M solutions of HCl or NaOH as required.

Halothane was vaporized by passing 4 L./min. of oxygen through a calibrated Fluotec vaporizer (Cyprane Ltd., England) and delivered to the cat in a continuous flow. The same technique was used to equilibrate mammalian ringer solutions at various concentrations. Once equilibration was achieved the solution was passed continuously through a plastic cup resting on the surface of the cuneate nucleus. The five-barrel micropipette penetrated the tissue through the centre of this cup (Fig. 2).

Gases such as halothane are rapidly removed from the tissue by its blood supply, at a rate which can reasonably be assumed proportional to the tissue concentration (y). The basic diffusion equation is then

$$dy/dy = D (d^2y/dx^2) - Ky$$

where D is the appropriate diffusion coefficient, and K the coefficient of removal.

In the steady state, this equation becomes

$$D (d^2y/dx^2) - Ky = 0.$$

For a semi-infinite solid, and a constant surface concentration (y_0), the solution is

$$y/y_0 = \exp (-x \sqrt{K/D})$$

(Crank, J. *The Mathematics of Diffusion*. London: Oxford University Press, 1956, p. 124).

If y_0 is the concentration of gas in the superfusing saline, a correction is required for the saline-brain partition coefficient (α). Then,

$$y/y_0 = \alpha \cdot \exp (-x \sqrt{K/D}).$$

Assuming complete equilibration between the blood and the tissue, we have

$$K = \beta \dot{V} \quad (\beta \approx 1/\alpha),$$

where β is the steady state partition coefficient between brain and blood, and $\dot{V}$ is the rate of blood flow (for the brain the mean value of $\dot{V}$ is 50 ml./100gm./min. or 0.008 ml./gm./sec.). For halothane, $\alpha = 2.6$ and $D = 10^{-5}$ cm./sec. We can therefore easily estimate the likely concentration of halothane at various depths, as a percentage of the concentration in the superfusing fluid.

Values of y/y_0 for different values of x are plotted in Fig. 2. One can see that within the range of depth where most of the cuneate cells were recorded (shown by arrow), the halothane concentration is likely to be within 75–150 per cent of the surface concentration.

ELECTRONIC EQUIPMENT—ELECTRODES

Recording

The recording was made in the cuneate nucleus, the medial lemniscus, and the superficial radial nerve. The signals were fed into a cathode follower and then into a Tektronix 122 AC preamplifier with a time constant of 2 msec. and a 502 Tektronic cathode ray oscilloscope.

The recordings from the medial lemniscus and the superficial radial nerve were made with bipolar silver electrodes (the nerve was crushed between them for monophasic recording); the signals were led on the same electronic circuit except for the cathode follower omitted for these recordings.

The action potentials were displayed on the oscilloscope and photographed with a Grass Camera C4. For single unit analysis the rate of discharge was also integrated and recorded on paper by an Oscillo/riter (Texas Instruments Inc.).

For the analysis of interspike intervals, burst discharges, and post-stimulus histograms, the gated action potentials were fed into a 100-bin digital computer (Ferch Electronics)¹⁸ and simultaneously displayed on a second oscilloscope; the histograms were recorded on paper as described above.

Compound action potentials on single or superimposed sweeps were photographed. Their width, height, and latency were analysed immediately after the experiments.

Stimulation

Stimulating electrodes were placed on the superficial and median nerves, the medial lemniscus, the pyramidal tract (in a few experiments they were placed on the somatosensory cortex) and the cuneate nucleus. Peripheral nerves and the medial lemniscus were stimulated with bipolar silver electrodes delivering electrical pulses of .02 msec. duration at a variable voltage and frequency. The pyramidal tract was stimulated with one-per-second bursts of 3 to 6 pulses (0.5 msec. duration) at a frequency of 500 to 1000 per second, delivered through bipolar silver electrodes. The voltage was variable; single pulses of 1 to 2 msec. duration were used in some experiments.

A special switch was used to record from or stimulate the cuneate nucleus through the central barrel of the micropipette. A 5 to 20 M Ω resistor in series with the electrode reduced the effect of changes in electrode impedance to less than 10 per cent of the total impulse.

The stimulators were the Grass SD 5 and Tektronix combination 162 and 161 wave-form and pulse generators. In a few experiments a special arrangement was used to deliver triangular pulses of variable rate of rise (ramps), to study the rate of accommodation of afferent terminals.

A four-channel polarizing unit controlled the iontophoretic release of the various drugs. The iontophoretic currents generated in these circuits were measured with an electronic galvanometer (John Fluke Co.). The cat was grounded with a silver chloride electrode attached to the muscle of the neck.

Preparation of the five-barrelled micropipettes

The electrodes were drawn out with a commercial puller (Narishige) and their tips broken to a 5 to 10 μ diameter, then immersed in distilled water and boiled until all air had been expelled from the various barrels. Once the electrode was at room temperature the water was sucked from each barrel with the aid of a small polyethylene tube. The central and one lateral barrel were filled with 2.7 M NaCl; the other three were filled with various combinations of drugs according to the experiment. Colour codes were used to identify these drugs. The electrodes were placed in special holders with their tips immersed in water, then placed in a dark box which was kept at 4 to 10°C. Twenty-four to forty-eight hours were allowed for the diffusion of the drug to the tip of the electrode.

These pipettes were used in no more than two different animals and stored for no more than one week. The resistance of each barrel was measured and recorded before and after its use.

Animal care: conduct of the experiments

Special care was taken to maintain the cats in good general condition during the experiments. Most animals had a mean blood pressure of 90 mm. Hg. Hypotension was corrected when indicated by (a) continuous intravenous administration of noradrenaline, (b) correction of metabolic acidosis with sodium bicarbonate, (c) oxygen-enriched respiratory input, (d) plasma expanders (Subtosan, Poulenc) and as fast and careful surgical preparation as was possible. Whenever the arterial blood pressure could not be maintained over 60 mm. Hg the experiment was discontinued.

Two paralyzing agents were used, tubocurarine and succinylcholine. Tubocurarine was given in single doses of 0.5 to 1 mg./kg. every 30 to 60 minutes during the first six hours. After this time further doses required were negligible. Unfortunately, tubocurarine lowered the blood pressure below 90 mm. Hg in most animals, thereby increasing the need for vasopressors. Furthermore, tubocurarine depressed the afferent terminals when given systemically or iontophoretically. (Work on this subject is in preparation.)

Succinylcholine was given by continuous intravenous injections of a solution of 1 mg./ml., at a rate of 0.2 to 0.5 ml./min. It offered some advantages over tubocurarine. The blood pressure was well maintained, while the experimental results showed no effect on synaptic transmission. (Work in preparation.)

RESULTS AND DISCUSSION

It is the present belief that all anaesthetics depress synaptic transmission through the same mechanism.¹⁹⁻²² However, our experimental evidence indicates that although synaptic transmission is disrupted by the various anaesthetics investigated, their site and mode of action appear to be different.

Procaine depressed the axon, the afferent terminals, and the cell soma membrane equally and in a way consistent with its non-specific activity as a blocker of Na conductance.²⁵ Nembutal was more effective in blocking the post-synaptic membrane but it also happened to have some of the properties of procaine. Halothane, on the other hand, had no depressant effect on the pre- or post-synaptic elements; on the contrary it seems to excite them. It depressed synaptic transmission along the dorsal columns-medial lemniscus pathway but not the pyramidal tract-internuncial system. (Work in preparation.)

The use of glutamate as an excitatory agent to study the effect of anaesthetics on the post-synaptic membrane is justified even if its function as a neuro-transmitter has not been established. The effect of this amino acid in the cuneate nucleus seems to be restricted to specialized receptors in the post-synaptic membrane. Thus, suppression of its excitatory action can be considered a post-synaptic effect.

The excitability in the afferent terminals is determined by changes in the size of the antidromic spike as recorded in a peripheral nerve. GABA and glycine depress action potentials originated on these endings.²³ Halothane has no significant effect upon these structures.

Transmission through CNS synapses can be depressed by conditioning pulses applied to inhibitory pathways. Nembutal depresses while halothane facilitates this inhibition.²⁴

The dorsal columns-medial lemniscus pathway is not involved in pain perception nor in the conscious state, two functions that are depressed in the anaesthetized state. However, synaptic transmission in the dorsal column nuclei is of importance in the study of anaesthetic drugs because of its situation in the first relay nucleus of a sensory pathway. Moreover, in this synapse we can identify with certainty afferent terminals, interneurons, and relay cells, as well as its

inhibitory input, an important experimental advantage for the simultaneous study of the action of anaesthetics on the various synaptic elements.

The effect of anaesthetics on synaptic transmission has been investigated before at the myoneural junction,²⁶ the sympathetic ganglion,²⁷ and the monosynaptic reflex in the spinal cord.²⁸ The information derived from those experiments is of great value, but it needs to be compared with other CNS areas, especially if they have some of the complexity suspected to exist in higher neuronal circuits involved in the conscious state.

These are preliminary observations of which a more detailed report will be made elsewhere. They demonstrate the value of the experimental preparation just described as a useful tool for the study of the mechanism of action of anaesthetics and other compounds on a synapse in the central nervous system.

SUMMARY

A description has been presented of a combination of techniques to study the effects of anaesthetics on synapses in the cuneate nucleus. Procaine, nembutal, and halothane depressed synaptic transmission but through three different mechanisms. The use of the techniques presented in this paper may help to explain the neuropharmacology of anaesthetic agents.

RÉSUMÉ

Une série de techniques permettant l'étude des effets d'anesthésiques sur les synapses du noyau de Burdach ont été décrites. La procaine, le nembutal et l'halothane dépriment la transmission synaptique, chacune de ces substances selon un mécanisme qui lui est propre. Les techniques présentées dans ce travail devraient permettre une meilleure compréhension du mécanisme d'action nerveuse d'agents anesthésiques.

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