Pharmacology Biochemistry and Behavior, Vol. 3, pp. 127–131. ANKHO International Inc., 1975. Printed in the U.S.A.

BRIEF COMMUNICATION

A Method of Direct Chemical Brain Stimulation in Behavioral Studies using Microiontophoresis[1]

GEORGE K. AGHAJANIAN AND MICHAEL DAVIS

Yale University School of Medicine and The Connecticut Mental Health Center
New Haven, Connecticut 06508

(Received 19 February 1974)

AGHAJANIAN, G. K. AND M. DAVIS. *A method of direct chemical brain stimulation in behavioral studies using microiontophoresis.* PHARMAC. BIOCHEM. BEHAV. 3(1) 127–131, 1975. – A method of microiontophoresis for direct chemical brain stimulation in chronic, awake animals for behavioral studies is described. Carbachol-induced drinking was employed to test the method. Fluid-filled micropipettes (tip diameters: 5–15 μ) were stereotaxically implanted in the region of the nucleus of the diagonal band. Up to 3 weeks after recovery, ions could be ejected directly into the brain of awake animals by passing a direct current through the pipette. Iontophoretic ejection of carbachol in doses as low as 0.05 μg elicited drinking. This effect could be blocked by intraperitoneal injection of 0.5 mg/kg scopolamine. Passage of choline ions produced no detectable effect. The microiontophoretic technique allows direct chemical brain stimulation in chronic, awake animals without major changes in tonicity or volume that can occur with crystalline or fluid implants through cannulae. Additionally, the technique allows precise localization, precise control of dosage, and minimal damage at the site of stimulation.

Microiontophoresis Chemical brain stimulation Carbachol Scopolamine Drinking

CURRENTLY there is considerable interest in eliciting behavior by direct chemical stimulation of the brain [4, 6, 10, 11, 12, 13]. This general approach ultimately may help to establish the relationship between chemical neurotransmission and behavior.

In order to stimulate the brain chemically, a cannula is typically implanted in a specific region through which either crystalline compounds or compounds in solution are subsequently injected. While the cannula technique has been important in suggesting both chemical and anatomical specificity for a variety of behaviors, it is not without limitations. First, in relation to smaller nuclei (e.g., in rodent brain) tip diameters of conventional cannulae are quite large, thus precluding precise localization of chemical depositions as well as causing substantial damage in the area of interest. Second, with crystalline implants it is difficult if not impossible to specify the amount of the compound released at its site of action or to control the duration of action. Third, with fluid injections the pressure required to inject fluids may damage tissue near the cannula tip or produce seepage back up the cannula tract. The latter can be a serious problem if the cannula passes through one of the cerebral ventricles.

A technique that has been used for direct chemical stimulation of single neurons in the brain is microiontophoresis [3]. Basically a micropipette filled with a charged compound is placed in a specific area of the brain in an acute anesthetized or chronic immobolized preparation [5]. When an electrical current of the same polarity as that of the compound is passed through the pipette, the compound is ejected.

Microiontophoresis has several advantages over mechanical methods of injection. First, glass micropipettes can be pulled to extremely fine tip diameters (e.g., a few microns) allowing extremely precise localization and minimal damage by the tip. Second, minute and precise quantities of chemical can be ejected since ejection is controlled by a constant current. Third, ejection can be stopped quickly by reversing polarity. Finally, this technique involves the passage of selected ions rather than fluids or solids and thus avoids major changes in volume or tonicity. Using microiontophoresis it has been possible to influence the firing rate of single neurons within the brain and the technique has gained wide acceptance in the study of chemical neurotransmission. In addition, a macroiontophoretic method, using a gel-filled cannula chronically implanted in specific

[1] This research was supported by NIMH Grants MH-17871 and MH-14459, NSF Grant GB-23685, and the State of Connecticut.

brain sites [8] has been successfully used to elicit temperature changes.

The purpose of the present study is to evaluate whether microiontophoresis can be used in a chronic, awake preparation for the elicitation of behavior. To our knowledge, fluid-filled micropipettes have not hitherto been used to elicit behavior in chronic preparations. To make this test, carbachol-elicited drinking was chosen, since this has proved to be a rather robust and unambiguous measure of the effects of direct chemical stimulation of the brain.

METHOD

Animals

Twenty male, albino Sprague-Dawley rats (Charles River Co.) that weighed between 300–350 g were used. Following recovery from implantation all animals were allowed free access to food pellets and water prior to testing.

Preparation of Pipettes

Pyrex glass tubing (O.D. = 2 mm, I.D. = 1 mm), previously loaded with a few strands of fiberglass, was pulled to a fine tip using a Narishige micropipette puller. The tip was broken back to 5–15 μ under microscopic control and the pipette was filled with 1.0 molar carbachol chloride. The fiberglass allowed the tip to become filled rapidly by capillary action [14]. This direct filling method is convenient and ensures that the concentration of carbachol at the tip of the pipette is the same as in the rest of the pipette. Impedances (measured at 1000 Hz with a F. Haer Impedence Check Module) were typically 2–6 MΩ. The pipette was then scored, but not broken, 14–15 mm above the tip and placed in a bath of 0.9% saline for several hours to allow the solution in the pipette to come to room temperature.

Implantation

Animals were anesthetized with chloral hydrate and placed in a stereotaxic instrument. The skull was exposed by a midline incision and the skin and fascia retracted. Four 1/8 in. stainless steel screws (0-80) were placed into the skull to serve as anchors for later application of dental cement. A 25 mm length of 20 ga copper core wire, which had been presoldered to a 30 ga wire, was positioned on the skull by wrapping the thin wire several times around one of the screws. The pipette was then lowered stereotaxically through a burr hole over the intended area to the desired depth and cemented in place. Care was taken to not get any cement above the score mark. In most cases the coordinates, relative to bregma, were lateral −0, anterior −0 to 1.5 mm, depth −6.5 mm. These coordinates usually resulted in placement in the nucleus of the diagonal band at a frontal plane of A7890 according to the atlas of König and Klippel [9]. This site has been shown to be a positive site for carbachol-elicited drinking [15].

After the cement had set and the pipette was rigidly in place, it was broken off at the previously scored point. A short length (3–4 mm) of teflon insulated platinum wire, whose tip was bared of insulation (1 mm) was inserted to the shoulder of the pipette. The other end of this wire, which had previously been soldered to a 25 mm length of 20 ga wire, was then cemented in place, taking care not to get any cement into the pipette. Platinum wire was used to minimize the possibility of a reaction with the fluid in the pipette. The top of the pipette was then sealed by applying a few drops of melted paraffin with a small brush. Next, liberal amounts of dental cement were applied so that both 20 ga wires and the sides of the pipette were enclosed by dental cement. Access to the top of the pipette and distal ends of the wires was preserved. The process was aided by fashioning a ring of clay, about 1 cm high, around the entire assembly and then filling this ring with cement. This prevented the cement from flowing onto unwanted areas and greatly reduced the time necessary for this part of the implantation procedure. After the cement had hardened the clay ring was removed and the two 20 ga wires were clipped to extend about 7–8 mm above the top of the cement. Finally, a tiny hole was punched through the paraffin, using an 80 μ wire, so that the pipette was not entirely sealed. This hole proved to be very important since exploratory work indicated that if the pipette were completely sealed at its top, impedances rose sharply in vitro and it was difficult or impossible to pass current in vivo.

Testing

Twenty-four hr to 3 weeks after implantation, the animals were tested to determine whether iontophoretic ejection of carbachol would elicit drinking. Testing was conducted in a open box equipped with a water-filled graduated cylinder attached to a drinkometer circuit. Bubble-clips were attached to the two 20 ga wire leads on the animal through a 2 ft cable leading to a custom-built constant direct-current stimulator having a source voltage of 1250 V. Since carbachol is a positive ion, for ejection purposes, positive current was passed through the lead that went into the pipette; the circuit was completed through the skull lead. The experimental setup was thus similar to that used for electrical stimulation of the brain except that in this case the current would be carried by drug ions.

Calculation of Dose from Current

The following formula was used to calculate the amount of carbachol ejected [3]:

$$M = \frac{nIT}{FZ}$$

where M is the moles ejected, n is the transport number, F is Faraday's constant (9.65×10^4), Z is the equivalents/mole, I is the current in amps and T is the time of ejection in sec. If n, which is a measure of ejection efficiency, is assumed to be 1.0, then the calculation yields the maximum amount of drug that can be ejected by a given current for a given time.

Histology

For all animals the localization of the micropipette was determined by histological examination of 50 μ cresyl-violet-stained serial sections. The exact site of the pipette tip can be marked by making a small lesion prior to perfusion with fixative (5% phosphate buffered Formalin). This can be accomplished by passing a high anodal current (e.g. 20 μA) for a prolonged period (e.g. 20 min) while the animal is anesthetized prior to perfusion.

RESULTS

In all of the animals tested, microiontophoretic ejection

of carbachol produced changes in behavior. In some cases, particularly with high ejection currents, this was manifested by dramatic shifts in activity where the rat would begin to actively explore the test cage, often pausing to groom vigorously, and then become even more hyperactive, darting from one corner to another. This would often end in intense drinking or occasionally in seizures. In other animals, very low ejection currents resulted in a short period of well directed exploration which invariably led to sustained drinking. For example, Fig. 1 shows the results of experiments with an animal in which the tip of the micropipette was later histologically determined to be located in the nucleus of the diagonal band. Various ejection currents were applied for 2 min periods on each of several test days. Figure 1 shows that higher ejection currents resulted in shorter latencies and longer periods of drinking. At the highest current tested the effect was dramatic, with about 20 ml of water being consumed in a 13 min period beginning 15 sec after the current was turned off. A phase of exploratory activity, which occurred during the period of ejection at the higher currents always preceded the onset of drinking. At lower currents no behavioral effects were typically seen during the ejection period.

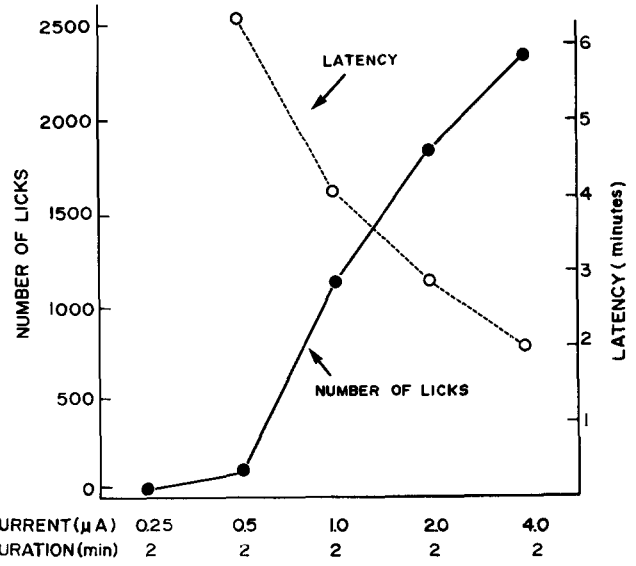

FIG. 1. Latencies to initiate drinking and the total number of licks during the 20-min period following ejection of carbachol at various currents (125 licks≅1 ml of water).

Using the formula mentioned earlier, the smallest amount of current that elicited drinking (0.5 μA for 2 min) would represent a maximal dose of 0.11 μg. Assuming an ejection efficiency of 48% (i.e., n = 0.48) rather than 100% (i.e. n = 1.0), based on previous empirical measures of efficiency of ejection from large micropipettes [1], the dose would be about 0.05 μg. In order to specify the dose more exactly, the transport number for a given substance and type of pipette must be determined empirically [3].

Similar dose-response curves were obtained from several other animals (n = 12). In each case, higher ejection currents resulted in shorter latencies and longer periods of drinking. Latencies ranged from about 27 sec to 15 min, depending on the dose and the electrode placement. While the number and distribution of placements were not extensive enough to specifically localize the most sensitive drinking area, our data suggested that the more anterior placements in the diagonal band resulted in longer drinking latencies or even no drinking, but only hyperactivity and seizures (n = 5). In several rats that had very short latencies, drinking was often followed by a peculiar languid posture where the animal would slither on its belly, similar to previous reports that have used cannulae in similar areas [2].

Several control procedures indicated that the behaviors elicited by microiontophoretic ejection of carbachol did not simply result from the passage of current. First, the elicited behaviors usually outlasted the period of ejection and frequently began after the positive ejection current had been turned off. Second, passage of negative current and the consequent release of chloride ions for extended periods of time did not produce any detectable changes in behavior. Third, in two animals that were implanted with micropipettes filled with 1.0 molar choline, massive ejection currents (e.g 600 μA-min) also failed to produce any changes in behavior. This indicates that the passage of positive current carried by choline ions, which resemble carbachol ions structurally but which are physiologically inactive, was not effective in eliciting behavior. Finally, Fig. 2 shows that drinking elicited by microiontophoretic ejection of carbachol could be blocked or diminished by intraperitoneal injection of 0.5 mg/kg scopolamine given 15 min prior to ejection.

In most animals it was possible to elicit drinking or alter behaviors associated with a particular placement over several days or sometimes several weeks. In general, however, response latencies tended to increase over days and in some cases, eventually it became impossible to pass current, perhaps because a gradual gliosis occurred around the tip. It is interesting in this regard that one of the best animals both in terms of stable latencies over time and in terms of absolute latencies (latencies to drink being about 150, 50 and 30 sec for ejections of 4, 8, and 16 μA-min, respectively) had its pipette directly in the anterior horn of the third ventricle, where gliosis would not occur.

Leakage of carbachol during non-stimulation periods did not seem to be sufficient to influence baseline levels of drinking. Thus, during test periods, virtually no drinking occurred in the absence of ejection. In addition, observation of daily water intake indicated that none of the rats, except one, showed an increase in post-implant drinking. In the latter case, there was a great deal of blood in the pipette tract, suggesting that the pipette was broken upon entry and consequently did leak significant amounts of carbachol. However, there may be other situations where leakage could become a problem (e.g., in a small, highly sensitive nucleus). This could be readily controlled by applying a retaining current of the opposite polarity [3].

Finally, all animals appeared in good condition after their implants and none deteriorated over subsequent weeks. Moreover, even though the dental cement caps were about 1 cm high, none of the animals lost their caps prior to sacrifice. Histological examiniation after anodal marking currents (20 μA, 20 min) revealed lesions approximately 0.1 mm in diameter situated at the tips of the electrode tracts.

DISCUSSION

The present results indicate that microiontophoresis is a

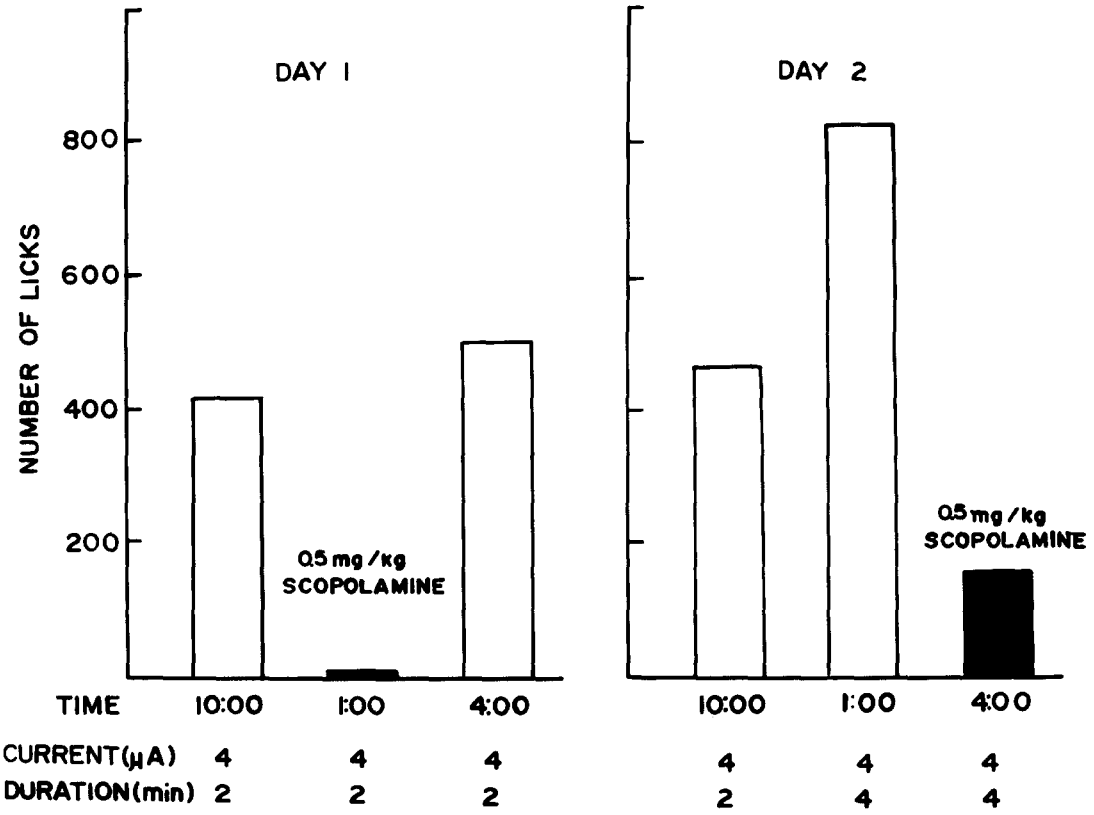

FIG. 2. Attenuation of iontophoretically elicited drinking by scopolamine. On each of two days, carbachol was ejected at 3 hr intervals (10:00 a.m., 1:00 p.m. and 4:00 p.m.) 15 min after interperitoneal injection of saline (white bars) or 0.5 mg/kg scopolamine (black bars).

feasible technique for eliciting behavior in chronic, awake animals. As outlined earlier its advantages include extremely precise localization and a more precise control of dosage than can be attained by other means of direct chemical stimulation of the brain. Localization of the electrode tip to a sphere 0.1 mm in diameter is readily accomplished through passage of an anodal current just prior to perfusion. With prolonged ejections at high currents there may be a considerable spread of iontophoretically applied drugs beyond the vicinity of the electrode tip [7]. However, the extent of the spread can be controlled by limiting the duration and intensity of ejection and through the use of retaining current during non-ejection periods.

There are of course some disadvantages to the iontophoretic method. One is that the compound to be tested must be stable enough not to be degraded between the time of implantation and testing. Another is that, once a micropipette is implanted, only one compound can be tested in that one area. In addition, with the passage of time it may become increasingly difficult to pass current. Perhaps some of these difficulties could be overcome by devising a system in which an outer guide were implanted and then micropipettes rather than inner cannulae were inserted just prior to testing [5]. Further, there is no reason why multibarrel pipettes couldn't also be implanted, allowing several compounds to be tested in highly localized areas. In conclusion, the microiontophoretic method can serve as an alternative to direct injection methods when there is a need for controlled ejections of drugs into small nuclei.

REFERENCES

1. Bradley, P. B. and J. M. Candy. Iontophoretic release of acetylcholine, noradrenaline, 5-hydroxytryptamine and d-lysergic acid diethylamide from micropipettes. *Br. J. Pharmac.* **40:** 194–201, 1970.
2. Crawshaw, L. I. Effect of intracranial acetylcholine injection on thermoregulatory responses in the rat. *J. comp. physiol. Psychol.* **83:** 32–35, 1973.
3. Curtis, D. R. Microelectrophoresis. In: *Physical Techniques in Biological Research, V. Electrophysiological Methods, Part A,* edited by W. L. Nastuh. New York: Academic Press, 1964, pp. 144–190.
4. Fisher, A. E. and J. N. Coury. Cholinergic tracing of a central neural circuit underlying the thirst drive. *Science* **138:** 691–693, 1962.
5. Frederickson, R. C. A., L. M. Jordan and J. W. Phillis. A technique for microelectrophoretic study of single neurons in the awake nonparalyzed cat. *Neuropharmacology* **12:** 77–81, 1973.
6. Grossman, S. P. Eating or drinking elicited by direct adrenergic or cholinergic stimulation of the hypothalamus. *Science* **132:** 301–302, 1960.

7. Herz, A., W. Zieglgansberger and G. Farber. Microelectrophoretic studies concerning the spread of glutamic acid GABA in brain tissue. *Expl Brain Res.* **9**: 221–235, 1969.
8. Kirkpatrick, W. E. and P. Lomax. Temperature changes following iontophoretic injection of acetylcholine into the rostral hypothalamus of the rat. *Neuropharmacology* **9**: 195–202, 1970.
9. König, J. F. R. and R. A. Klippel. *The Rat Brain – A Stereotaxic Atlas of the Forebrain and Lower Parts of the Brain Stem.* New York: Krieger, 1970.
10. Myers, R. D. Chemical mechanisms in the hypothalamus mediating eating and drinking in the monkey. *Ann. N.Y. Acad. Sci.* **157**: 918–931, 1969.
11. Miller, N. E. Chemical coding of behavior in the brain. *Science* **148**: 328–338, 1965.
12. Routtenberg, A. Intracranial chemical injection and behavior: A critical review. *Behav. Biol.* **7**: 601–641, 1972.
13. Singer, G. and R. B. Montgomery. Specificy of chemical stimulation of the rat brain and other related issues in the interpretation of chemical stimulation data. *Pharmac. Biochem. Behav.* **1**: 211–221, 1973.
14. Tasaki, K., V. Tsukahara, S. Itu, M. J. Wayner and W. Y. Yu. A simple, direct and rapid method for filling microelectrodes. *Physiol. Behav.* **3**: 1009–1010, 1968.
15. Terpstra, G. K. and J. L. Slagen. The role of the tractus diagonalis in drinking behavior induced by central chemical stimulation, water deprivation and salt injection. *Neuropharmacology* **11**: 807–817, 1972.