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Isolated Frog Brainstem Technique: Analysis of Neural Correlates of Mating Calling and Possible Use in Drug Studies

David Douglas Schaffer
Loyola University Chicago

ISOLATED FROG BRAINSTEM TECHNIQUE: ANALYSIS OF NEURAL CORRELATES OF MATING CALLING AND POSSIBLE USE IN DRUG STUDIES

by

David D. Schaffer

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VITA

The author, David Douglas Schaffer, is the son of Jane (Osborn) Schaffer and C. Elmer Schaffer. He was born December 27, 1944, in Hartford, Connecticut.

He attended elementary and junior high school in Kettering, Ohio. He was graduated from Fairmont High School, Kettering, Ohio, in May 1963.

He entered Denison University in 1963 and received the degree of Bachelor of Arts with a major in chemistry in June of 1967. In 1967 he entered the School of Veterinary Medicine, Tuskegee Institute. Tuskegee Institute awarded him the degree Bachelor of Science in Agriculture with a major in animal science in May 1970, and the degree Doctor of Veterinary Medicine in May 1971.

On June 22, 1968, he married Caroline Sue Brunsman.

Dr. Schaffer worked as a veterinarian at the Columbus Pet Hospital in Columbus, Ohio, during 1971 and 1972.

He was accepted by the Department of Pharmacology, Loyola University, Stritch School of Medicine in 1973 and was awarded an NIH Training Assistanceship. He was selected as an Arthur J. Schmitt Fellow for the academic year 1977-1978.

While attending Loyola University, he was elected

student representative to the Graduate Student Council of Loyola University Medical Center for the academic year 1978-1979. He was treasurer of the Graduate Student Council and the student representative to the Animal Care Committee from 1978 to 1979. In April 1979, he was inducted into Alpha Sigma Nu, the National Jesuit Honor Society.

Dr. Schaffer is a member of the American Veterinary Medical Association, the American Animal Hospital Association, and other professional organizations.

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CHAPTER I

INTRODUCTION

A. Objectives

One purpose of this research project was to quantitatively describe the normal neural correlates of mating calling activity of Rana pipiens pipiens as recorded from the isolated brainstem preparation and to provide a measure of the variability occurring among different preparations.

Another objective was to determine the effect of different temperatures on electrically evoked mating calling activity recorded from the isolated brainstem.

A final objective was to investigate the feasibility of using the isolated frog brainstem in drug studies of calling activity. To do this, the newly developed isolated frog brainstem technique of Schmidt (1976) had to be modified and further developed to permit the application and removal of drugs. Through the design and construction of a continuous flow-through tissue bath, recordings of neural correlates of electrically evoked mating calling activity could be made from a completely isolated frog brainstem when drugs were added to or removed from the bath.

B. General background information

Male Northern leopard frogs, Rana pipiens pipiens, were used in this research. Male leopard frogs produce a variety of vocalizations (Schmidt, 1968b; Mecham, 1971; Pace, 1974). Several types of calls are given frequently in a sequence by individual frogs and numerous different calls often are heard simultaneously from a chorus of frogs (Mecham, 1971; Pace, 1974). These calls include the mating call, release call, warning cry, and a variety of chuckle calls (Schmidt, 1968b; Mecham, 1971; Pace, 1974). Two of these calls, release calls and mating calls, are of special interest in this project.

1. Release calls - function

Although release calls are not studied in this research project, knowledge of them aids in understanding mating calls. Release calls play an important role in the mating behavior of leopard frogs. They are given by males as well as non-gravid females when males clasp them. This random clasping happens often during the relatively short breeding season of R. p. pipiens. The frogs often form breeding aggregations with high male densities. The males then engage in "scramble competition," actively moving about in search of females (Wells, 1977). They are attracted by the movement of any frog, male or female. Apparently unable to discriminate visually between males

and females, they clasp other males as well as females. The clasped male or non-gravid female gives a release call. The clasping male then releases his clasp. It is not known whether the frog releases because he hears the release call or because he feels the vibrations of the sides of the calling frog. Schmidt (1974a) presented evidence that, at least in the toad Bufo americanus, it is mainly the tactile effects rather than the auditory effects of the release signals that evoke releasing. When a sexually active male clasps a gravid female, no release call is given and clasping continues until oviposition is completed.

2. Mating calls - function

Just as the release call plays an important role during the breeding season, so does the mating call. The species specificity of the mating call is believed to be important in attracting conspecific gravid females, thereby serving as a premating isolation mechanism. This function of the mating call has been shown experimentally for two sympatric Australian hylids, Hyla ewingi and H. verreauzi (Loftus-Hills and Littlejohn, 1971) as well as for many other species but has not yet been shown experimentally for R. p. pipiens. Pace (1974) argued that since mating calls are "the most distinctive and conspicuous" calls of leopard frogs, they are thus "most likely to be

the ones involved in species discrimination and mate attraction. Furthermore, at least in R. pipiens, this call is the one given most frequently by lone males in the field, as would be expected of a mate-attracting call." The mating call is dependent upon the presence of testicular hormone (Schmidt, 1966b) and of the preoptic area (Schmidt, 1968a), providing more evidence that the call is indeed a mating call. Oldham (1974) has reported suggestive behavioral evidence favoring an attractive function for ranid mating calls.

3. Description of calls

The release call and the mating call are similar in that they both consist of a series of sound pulses and have approximately the same pulse rate. The release call has a short series of pulses. It is repeated rapidly, giving it a short interval between successive calls. The mating call lasts longer and has a longer intercall interval than the release call (Schmidt, 1968b, 1974c).

The mating call of R. p. pipiens has been described as a long pulsed call lasting over one second to as long as five or more seconds, with a pulse rate of approximately 20 pulses per second at 60°F. The number of pulses per call varies considerably but averages about 50 pulses (Pace, 1974). The pulse rate has been reported by other investigators at a number of different temper-

atures (Littlejohn and Oldham, 1968; Mecham, 1971; Brown and Brown, 1972).

4. Taxonomic importance of mating call

To the taxonomist, the species-specificity of the mating call is very important. The mating call is believed to be one of the main characteristics separating species within the Rana pipiens complex. Differences in the structure of mating calls of frogs within the complex enable frogs to be classified into several different species (Littlejohn and Oldham, 1968; Mecham, 1971; Brown and Brown, 1972; Pace, 1974; Frost and Bagnara, 1976). The mating call of R. p. pipiens has a characteristic length, pulse rate, number of pulses per call, as well as other characteristics that make it unique from the mating calls of other species within the complex.

5. Peripheral structures producing calls

The peripheral motor structures involved in producing a release call or a mating call are the same. These same peripheral structures are also involved in pulmonary respiration (Schmidt, 1966a). As with the frog, Peek, Youngren, and Phillips (1975) found marked similarities between respiration and vocalization in chickens. A detailed description of the peripheral events involved in calling and respiration in the frog have been provided

by direct observation of the larynx and other peripheral structures (Schmidt, 1965, 1966a).

a. Phases of calling and respiration

Both calling and pulmonary respiration consist of two distinct phases (Fig. 1 and 2). The first phase involves expiration of air from the lungs. This phase is silent during respiration and is termed the expiratory phase. During calling, sound is produced when the glottis opens, allowing air to pass the opposed vocal cords, causing them to vibrate. In calling, this initial phase is called the vocal phase (Schmidt, 1966a).

The second phase is called the inspiratory phase in both calling and pulmonary respiration. During this phase, air enters the lungs. This phase appears to be identical in both calling and breathing (Schmidt, 1966a).

b. Description of peripheral events producing a call

To produce a mating call or a release call, the expiratory muscles of the frog's body wall contract, increasing the intrapulmonary pressure. This increased pressure pushes air from the lungs whenever the glottis is open. Each time the glottis opens, the vocal cords become opposed at the midline. As air rushes past the vocal cords, the cords vibrate to produce sound. The

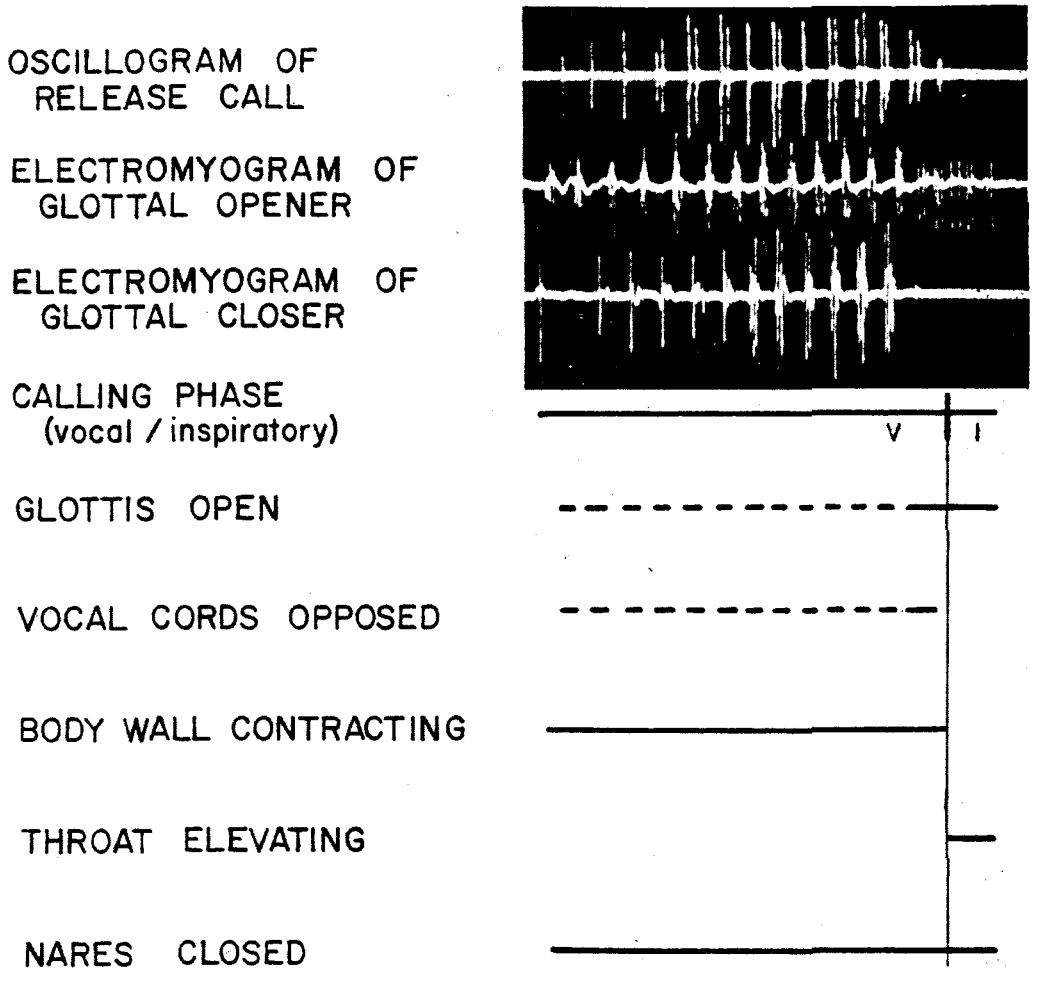

Fig. 1. Kinetochronograms of some important events involved in release calling of Rana pipiens pipiens. V = vocal phase of calling, I = inspiratory phase of calling. Vertical time lines in upper three traces occur at 100 msec intervals. (From Schmidt, 1973.)

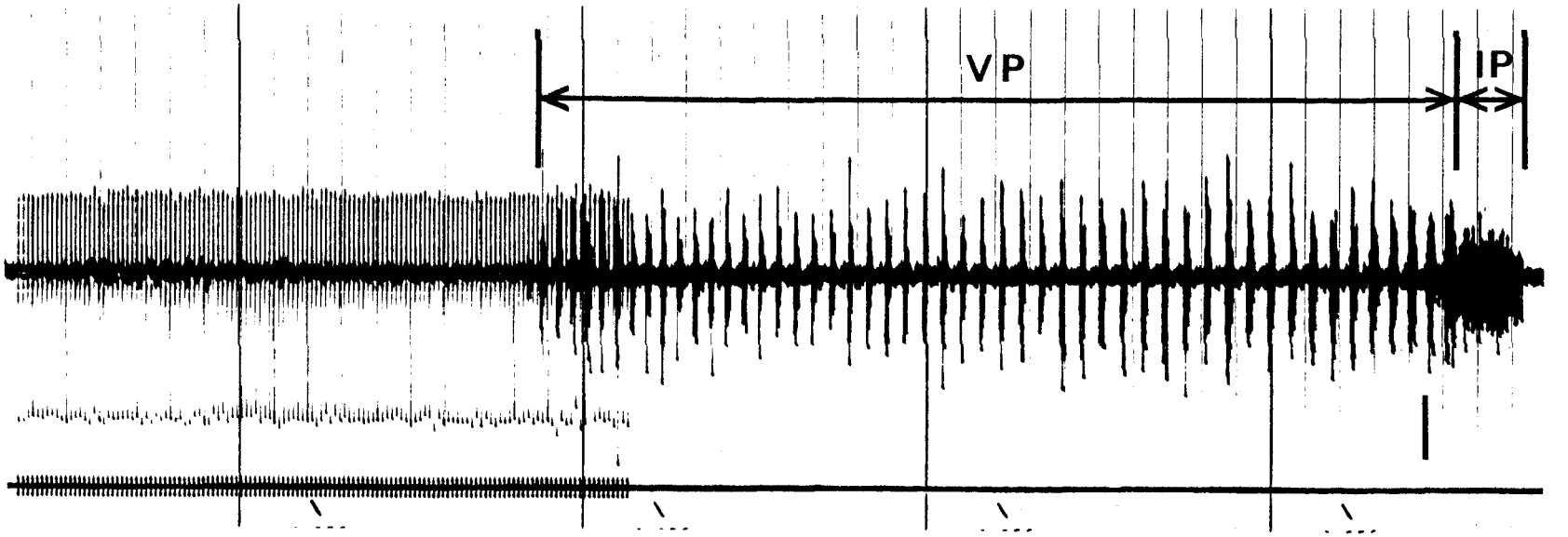

Fig. 2. Neural correlates of mating calling activity evoked by electrical stimulation of the anterior preoptic nucleus of the isolated frog brainstem. Upper trace showed a pulsed vocal phase (VP) followed by a tonic inspiratory phase (IP) as recorded from the left short laryngeal nerve. Lower trace was the stimulus monitor. The bolder vertical time lines occur at one second intervals. Amplitude scale (lower right) = 100 μV.

glottal opener and glottal closer muscles in the larynx work in opposition to open and close the glottis rhythmically. This produces the pulses of sound sometimes referred to as a trill. The frog's mouth and nares remain closed during this vocal phase. The air that leaves the lungs during this period inflates the paired vocal sacs of the frog (Schmidt, 1966a, 1973).

The inspiratory phase of calling immediately follows the vocal phase. With the mouth and nares still closed, the frog elevates his throat causing air in the vocal sacs to return to the lungs through the tonically open glottis. This phase is silent since the vocal cords are unopposed (Schmidt, 1966a, 1973).

c. Studies of peripheral motor events producing calls

Studies of the peripheral motor events producing calls serve as a basis for much of the subsequent studies of the central control of calling. A knowledge of the peripheral motor events is needed for an understanding of what the central nervous system must accomplish to produce calls.

Movement of the more critical structures involved in leopard frog calling were studied and described by Schmidt (1965, 1966a, 1972) (Fig. 1). He demonstrated that laryngeal calling movements, as well as calling

movements of the throat and sides, are correlated with the gross temporal patterns of a sound oscillogram of a call. Based on a detailed analysis of the action of the intrinsic muscles during release calling, he concluded that an electromyogram of one of the intrinsic laryngeal muscles provided an "ideal event with which to correlate central activity during calling" (Schmidt, 1972). He summarized many of the important events involved in leopard frog calling in the kinetochronogram shown in Fig. 1 (Schmidt, 1973). This figure shows how the electrical activity of two intrinsic laryngeal muscles, one a glottal opener and the other a glottal closer, correlate with a sound oscillogram of the actual call as well as with several of the peripheral motor events producing the call.

As in Schmidt's research, Youngren, Peek, and Phillips (1974) related muscle activity with sound production in chickens. They found a correlation between the electromyograms of the two opposing tracheal muscles and repetitive vocalizations.

Schmidt (1974b) identified four advantages to representing calling behavior by just two electromyograms. Laryngeal electromyograms 1) simplify calling into two muscle movements that are easily studied, 2) provide an objective method of monitoring calling, 3) are more directly linked to the central mechanism of calling than are other activities of calling, and 4) can be recorded

even when lesioning prevents the actual production of calling sounds.

The electromyograms of an intrinsic laryngeal muscle that opens the glottis of R. p. pipiens show mating calling activity because

> First, they show the same basic patterns of activity found in the electromyograms recorded during tactilely evoked release calling in intact frogs. Second, both release calls and mating calls, when elicited in intact frogs by natural stimuli, are pulsed and are followed by an inspiratory movement, and their gross temporal patterns differ only in call duration and intercall interval. Third, the muscle activity. . .(shown by the electromyogram of the glottal opener muscles). . .occurs only in response to brain (preoptic area) stimulation. Fourth, electrical stimulation of the preoptic area can evoke mating calls that differ only slightly (oscillograms tend to be shorter than, and therefore more nearly the duration of, release calls) from mating calls evoked in intact frogs by natural stimuli. Fifth, ablation of the preoptic area abolishes mating calling but not release calling evoked by natural stimuli. . .(Schmidt, 1974b).

Schmidt (1974c, 1976) later found that anuran calling behavior could be monitored directly from either the trigeminal tegmentum or the short laryngeal nerves and that these recordings show the same gross temporal patterns as those shown in electromyograms of the glottal opener muscles in acute leopard frogs.

6. Central mechanism of sound production

Evidence shows that some calling behavior patterns are fixed action patterns, i.e. they are coded in and controlled by the central nervous system in the frog as well

as other species of both vertebrates and invertebrates. These patterns occur independently of peripheral feedback as evidenced by the isolated frog brainstem technique (Schmidt, 1976). Ongoing research by Schmidt and others is aimed at understanding the specific central mechanisms involved in calling.

The studies of David Bentley on the neurobiology of cricket song (Bentley and Hoy, 1974; Bentley, 1977) provide an excellent example of the central programming of song patterns in invertebrates. Bentley has shown that the two anterior thoracic ganglia generate the calling song and that these ganglia can generate the pattern independent of sensory input. The calling song is the most common cricket song. It is sung by males to attract sexually receptive females. The calling song pattern is considered species specific since each cricket species has a distinctive calling song pattern. The song patterns of different cricket species are not learned behavior but are encoded in the cricket's genes (Bentley and Hoy, 1974).

Vocalization studies of representatives of every class of vertebrate show the existence of central pattern generators. Vocalizations were studied in reptiles (e.g. lizard - Kennedy, 1975), mammals (e.g. squirrel monkeys - Jürgens and Pratt, 1979), and several species of birds (e.g. turkeys - Anschel, 1977; redwinged blackbirds - Brown, 1971; canaries - Nottebohm, Stokes, and Leonard,

1976; chickens - Phillips and Peek, 1975). Even the sound production of the toadfish Opsanus beta (Demski and Gerald, 1972) and Opsanus tau (Fine, 1979) has a central pattern generator. The toadfish produces sound by contraction of the muscles of the swim bladder rather than by true vocalization.

Considerable work has been reported on the central mechanisms involved in repetitive vocalizations in chickens. Electrical stimulation of the brain in anesthetized chickens (Peek and Phillips, 1971) and in awake, unrestrained chickens (Phillips, Youngren, and Peek, 1972) revealed an apparently continuous area in the brain extending from the preoptic region to the most posterior portion of the medulla that is involved in vocalization. Phillips and Youngren (1976) also used electrical stimulation of the midbrain area, coupled with multiunit recordings of medullary neurons, to investigate the central generation of chicken vocalization patterns.

The first modern work on the central mechanisms of frog calling is credited to Aronson and Noble (1945). They investigated the effects of brain lesions on several aspects of leopard frog behavior.

Schmidt made strides in the study of the central mechanisms of frog calling when he discovered that release calling (Schmidt, 1966a) and mating calling (Schmidt, 1968a) could be evoked in both acute and chronic

frog experiments by electrical stimulation of the brain. He found this evoked calling to be similar to or nearly indistinguishable from calling evoked by natural stimuli. Release calling was evoked by electrical stimulation of the trigeminal tegmentum of the medulla (Schmidt, 1966a). Mating calling was evoked by electrical stimulation of the preoptic area (Schmidt, 1968a). Schmidt (1966a, 1968a, 1971, 1973) combined his techniques for electrical stimulation of calling with transection, ablation, and lesion studies of the brain.

Schmidt made further advances with the development of techniques for studying neural correlates of electrically evoked calling activity recorded from the completely denervated (Schmidt, 1974b, 1974c) and the completely isolated (Schmidt, 1976) frog brainstem. These studies confirmed his hypothesis that the central generation of the calling pattern, especially the mating calling pattern, is independent of peripheral feedback.

7. Model of the central mechanisms of frog calling

Based on his studies of the central mechanisms of frog calling, Schmidt (1971, 1973, 1974c, 1976) has constructed a series of tentative models of the central calling-pattern generator. The most recent model, a model of hormonally evoked mating calling, appears in Fig. 3 (Schmidt, 1976). The models show several similar-

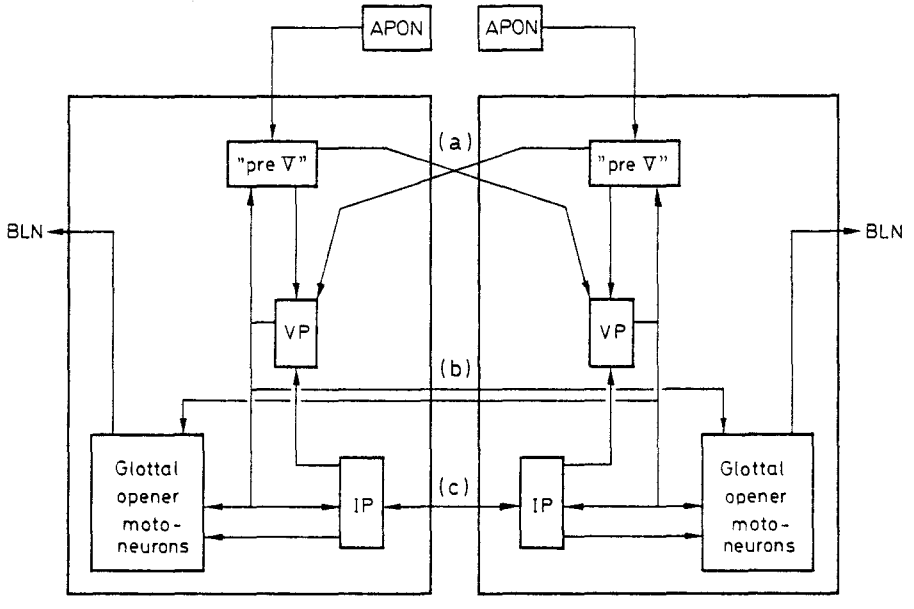

Fig. 3. Model of hormonally evoked mating calling. APON = anterior preoptic nucleus, "pre V" = region of the pretrigeminal nucleus, VP = vocal phase generator, IP = inspiratory phase generator, BLN = short (brief) laryngeal nerve. Connection (a) occurs at level of inferior colliculi and cerebrum; connection (b), at level somewhere posterior to cerebellum; connection (c), at level of hypoglossal nuclei. (From Schmidt, 1976)

ities to published models of the central mechanisms of vocalization in chickens (Phillips and Peek, 1975; Peek, Youngren, and Phillips, 1975) and the central mechanisms of sound production in toadfish (Demski, Gerald, and Popper, 1973).

a. Medullary calling circuits

Schmidt postulates that the neural circuits responsible for the generation of the basic motor patterns of calling, i.e. a pulsed vocal phase followed by a tonic inspiratory phase, are restricted to the medulla and possibly the immediately adjacent isthmus. This area is represented by the two large rectangles in Fig. 3. The anterior edge of this area is at approximately the anterior edge of the secondary visceral nuclei. The posterior limit of the rectangle is at about the posterior edge of the hypoglossal nuclei (Schmidt, 1976).

The medullary calling circuits function as a single basic vocal mechanism for the generation of the whole range of calling patterns. The various inputs to the medullary circuits determine the appropriate call to be produced (Schmidt, 1974c). The medullary calling pattern generator produces both release calling and mating calling. Both of these calling patterns consist of a pulsed vocal phase followed by an inspiratory phase. A single sound generating mechanism is also postulated in

models of chicken vocalizations (Phillips and Peek, 1975; Peek, Youngren, and Phillips, 1975).

Schmidt's studies of neural correlates of electrically evoked calling in the isolated frog brainstem convinced him that each side of the brain contains the mechanisms necessary for the generation of calling. He found that he could electrically evoke normal mating calling activity from either side of an isolated brainstem split midsagitally (Schmidt, 1976). Interconnections between the two sides synchronize activity in the unsplit brainstem.

1) Pretrigeminal nucleus

Schmidt (1974c) has identified the "pretrigeminal nucleus" (Fig 4) in the trigeminal tegmentum of the anterior medulla as an important part of the calling pattern circuits. He postulated that the region of the pretrigeminal nucleus serves as a sensory correlation area. As such, this area may receive and analyze a variety of sensory inputs and then determine the appropriate call (Schmidt, 1971, 1973). More recent evidence suggests that the pretrigeminal nucleus may also be involved in motor coordination of calling movements (Schmidt, 1974c). The most recent model of calling emphasizes close interaction between the region of the pretrigeminal nucleus and the more posterior portions of the medullary calling

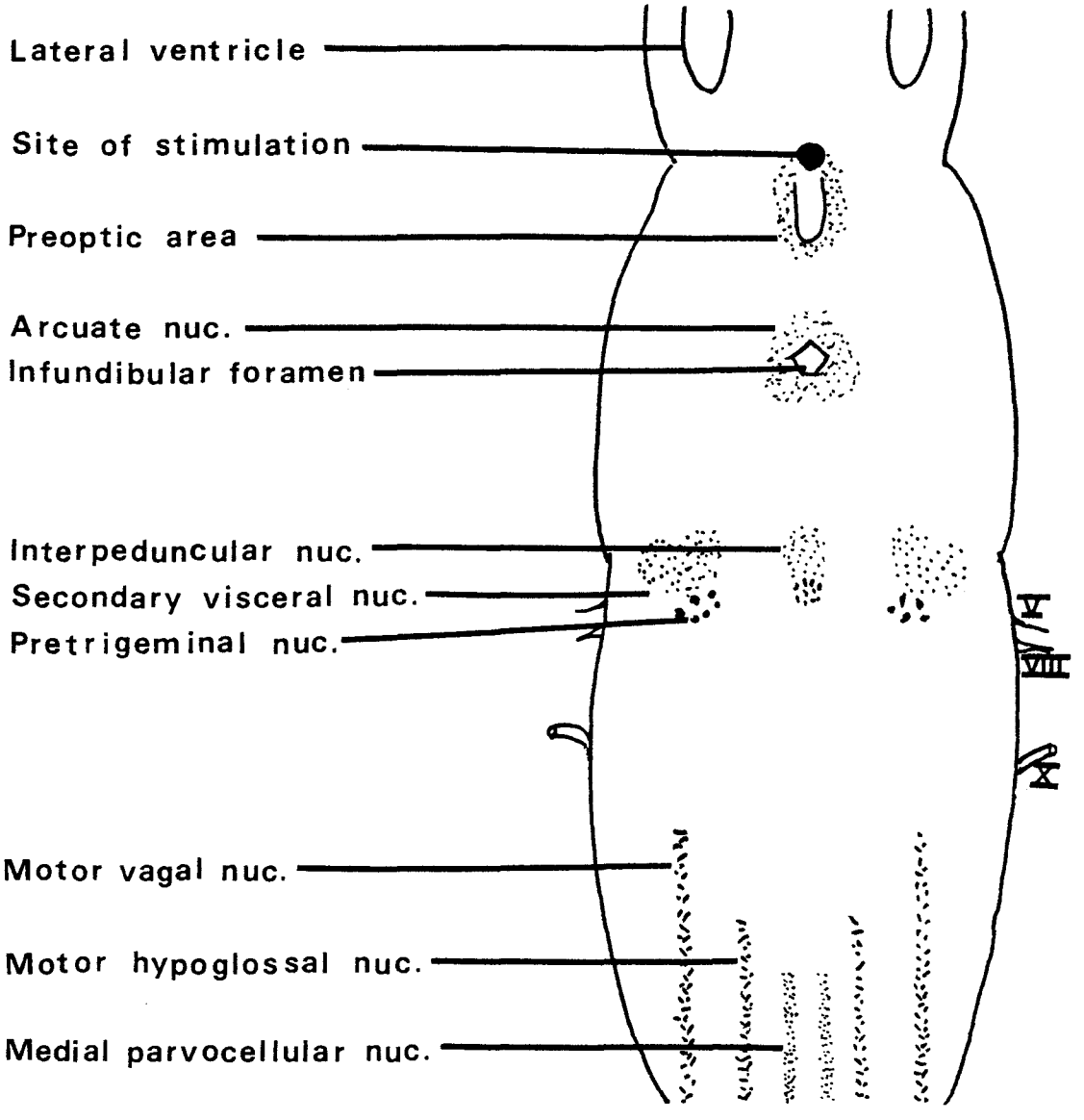

Fig. 4. Diagramatic view of frontal section through frog brain showing (projected on the same horizontal plane) some of the structures mentioned in the text and a few important landmarks. Black midline circle marks site for electrical stimulation of the anterior preoptic nucleus to evoke mating calling activity. (Adapted from Schmidt, 1974c)

circuits in the generation of calling patterns (Schmidt, 1976).

Bilateral lesions of the pretrigeminal nuclei abolish both release calling and mating calling (Schmidt, 1974c). Neural correlates of electrically evoked mating calling can be recorded directly from the pretrigeminal nucleus (Schmidt, 1974c, 1976). Electrical stimulation of the pretrigeminal nucleus results in the generation of the basic calling patterns (Schmidt, 1974b, 1974c).

Calling activity evoked by electrical stimulation of the pretrigeminal nucleus or by stimulation of other areas within the medullary calling circuits generally shows some imposition of the characteristics of the electrical stimulation. The calling activity usually stops as soon as stimulation is discontinued. The pulse rate of the vocal phase shows a direct pulse-to-pulse correlation with the pulse rate of the electrical stimulus. Schmidt (1974b, 1974c) interprets this as being due to the fact that the electrical stimulus is being applied directly to some portion of the integrative circuits of calling. Calling triggered by electrical stimulation of the anterior preoptic area which is outside of the medullary pattern generators results in more natural calling activity (Schmidt, 1974b, 1974c, 1976).

Demski and Gerald (1972) and Fine (1979) found the same type of one-to-one response when they evoked

sound production in toadfish by electrical stimulation in the middle and posterior portions of the medulla and in the cervical spinal cord. More natural sounds were evoked by electrical stimulation in the anterior portion of the medulla and in the midbrain. Jürgens and Pratt (1979) found that natural sounding, species specific vocalizations can be evoked in squirrel monkeys by midbrain stimulation. Stimulation of the lateral medulla, on the other hand, produced vocalizations of an artificial character. They interpret this as evidence that motor coordination occurs in the lower brainstem.

2) Posterior portions of the medullary calling circuits

Schmidt identifies two areas in the posterior medulla at the level of the obex that are important for calling. The first area consists of small cells medial to the hypoglossal nucleus (Fig. 4). Lesion data implicates this area as an inspiratory phase generator (Schmidt, 1971). The inspiratory phase generator is responsible for the inspiratory phase of both calling and respiration. Lesions in this area modify or abolish calling movements of the larynx.

The other important area of the posterior medulla is the area of the motoneurons innervating the muscles involved in calling such as the intrinsic laryngeal

muscles. In the case of the isolated brainstem preparation described in Chapter II, these include the glottal opener motoneurons of the vagal motor nuclei (Fig. 4) which leave the brain in the laryngeal nerves since it is the activity of these motoneurons that is recorded during electrically evoked mating calling (Schmidt, 1976).

Schmidt's research suggests that the region of the obex is involved in the integrative calling circuits. He found that neural correlates of calling could be recorded directly from the region of the obex during electrically evoked release calling and mating calling (Schmidt, 1974b). In addition, low threshold electrical stimulation in the region of the obex can evoke pulsed activity that is identical to the pulse rate of the electrical stimulation.

The exact location within the medulla of the vocal phase generator in Schmidt's model of calling in Fig. 3 is not known. Schmidt believes it is closely associated with the generation of the expiratory phase of respiration (Schmidt, 1976). He claims both the vocal phase generator and the inspiratory phase generator project to the glottal opener motoneurons since both phases were recorded from the short laryngeal nerves during electrically evoked calling.

b. Anterior preoptic nucleus

The anterior preoptic nucleus within the preoptic area (Fig. 4) and its posterior projections to the medullary calling circuits are necessary for the generation of mating calling. Experimental ablations and lesions of the preoptic area or its posterior projections abolish mating calling (Schmidt, 1968a, 1973). Neural correlates of mating calling were evoked by electrical stimulation of the anterior preoptic nucleus in the denervated brainstem (Schmidt, 1974b) and in the completely isolated frog brainstem (Schmidt, 1976). Neural correlates have been evoked by preoptic stimulation in the isolated brainstem of juvenile R. p. pipiens as early as 14 days following transition from gill respiration to pulmonary respiration (Schmidt, 1978).

Schmidt demonstrated that electrically evoked mating calling is a fixed action pattern independent of the eliciting stimulus. Electrical stimulation of the anterior preoptic nucleus triggers mating calling activity that goes on to completion even after termination of the electrical stimulation (Fig. 2). Sometimes the mating calling activity actually begins after the electrical stimulation has ceased (Schmidt, 1968a, 1974b, 1974c). The pulse rate of the vocal phase of electrically evoked mating calling shows no relationship to the pulse rate of

the eliciting electrical stimulus (Schmidt, 1968a, 1974b, 1974c).

The independence of the pattern of electrically evoked mating calling from the eliciting electrical stimulus is interpreted by Schmidt as evidence that the anterior preoptic nucleus lies outside the integrative calling pattern generator circuits. He hypothesizes that "the preoptic area is essentially another receptor area completely outside the main circuitry involved in the sensory correlation of the stimuli normally evoking calling and in the motor coordination of calling patterns" (Schmidt, 1974c). He further hypothesizes that electrical stimulation of the preoptic area results in a relatively normal "unpatterned" input which triggers the medullary calling pattern generators. This is indicated in his models of the central mechanisms of calling (Schmidt, 1974c, 1976) by placing the anterior preoptic nucleus outside the large rectangle that indicates the limits of the pattern generation circuits (Fig. 3).

Other investigators have evoked mating calling in Rana pipiens (Wada and Gordman, 1977) and in treefrogs Hyla arborea savignyi (Knorr, 1976) by stimulating electrodes chronically implanted in the preoptic nucleus.

Androgen receptors, postulated to exist in the anterior preoptic nucleus, have a role in the model of calling circuits proposed by Schmidt (1968a, 1976). When

they are stimulated by androgen during the mating season, the receptors activate the more posterior medullary calling circuits.

Androgens have been shown to function in calling because castration abolishes mating calling and because the injection of Rana pituitaries (pars distalis) will bring male R. p. pipiens, Hyla cinerea, and H. versicolor into calling condition. The pituitary injections probably act indirectly by stimulating the production of testicular hormone that then activates the preoptic androgen receptors (Schmidt, 1966b).

Evidence for the existence of androgen receptors in the anterior preoptic nucleus is contained in a number of recent studies using steroid autoradiography. After the injection of tritiated testosterone, labeled cells were found in the anterior preoptic area of R. p. pipiens (Kelley, Lieberburg, McEven, and Pfaff, 1978) and of Xenopus laevis (Kelley, Morrell, and Pfaff, 1975; Kelley, 1978). They also injected tritiated estradiol into Rana pipiens and Xenopus laevis. In like manner, they found concentrations of labeled cells in the anterior preoptic area. These investigators suggest that testosterone is metabolized to estradiol, explaining the appearance of labeled cells in the preoptic area after injection of 3 H-estradiol. Other evidence suggests that the distribution of sex steroid retention sites of anuran brains

follows a pattern common in other vertebrates (Morrel, Kelley, and Pfaff, 1975; Morrel and Pfaff, 1978; Kelley, 1978; Kim, Stumpf, Sar, and Martinez-Vargus, 1978; Arnold, Nottebohm, and Pfaff, 1976; Kelley and Pfaff, 1976).

CHAPTER II

MATERIALS AND GENERAL METHODS

A. Animals

1. Care and handling

Male Northern leopard frogs, Rana pipiens pipiens, purchased from Hazen, Inc. in Alburg, Vermont, and from Mogul-Ed in Oshkosh, Wisconsin, were used. They were kept in laundry sinks and fed crickets that had been dusted with a powdered vitamin-mineral supplement (Schmidt and Hudson, 1969). The frogs were kept under continuously operating fluorescent ceiling lights. They were maintained on the premises under these controlled conditions for several months before use in research to insure their being healthy and fully recovered from the stress of shipping.

2. Taxonomy

The taxonomy of leopard frogs has long been a subject of debate and has importance to this research project. Leopard frogs contain a number of populations considered by some as separate species and by others as subspecies.

These populations are collectively referred to as the Rana pipiens complex. It is imperative that all frogs used in this research give the same calling pattern lest variabilities in calling patterns are erroneously credited to experimental effects rather that species variability. R. p. pipiens is the only race or species of the Rana pipiens complex used in this project.

Rana pipiens is the most widely distributed and, in some areas, the most abundant North American frog. Individuals from a single locality or local population are generally fairly homogeneous. Different populations can, however, be distinctly different. The differences between these populations are often of a magnitude as great as those separating species (Moore, 1975). The debate centers on whether the Rana pipiens complex is actually one or several species.

Among populations in the Rana pipiens complex, there are distinct differences in the duration of the call (i.e. vocal phase length), the pulse rate, the number of pulses per call, as well as other parameters (Brown and Brown, 1972; Mecham, 1971; Littlejohn and Oldham, 1968; Frost and Bagnara, 1976; Pace, 1974). Those frogs giving a mating call with a long (greater than one second), many pulsed trill are called R. p. pipiens by Pace (1974). This has also been described as the Northern type of mating call (Littlejohn and Oldham, 1968; Brown and Brown,

1972; Pace, 1974).

Besides mating calling characteristics, other morphological characteristics have been used to distinguish individuals in the Rana pipiens complex. R. p. pipiens has a continuous dorsolateral fold extending in an unbroken line from just behind the eye to the area where the leg joins the body (Pace, 1974). The folds are broad and low and do not contrast strongly with the ground color (Brown and Brown, 1972; Pace, 1974; Frost and Bagnara, 1976). The males usually have Mullerian ducts (i.e. vestigial oviducts) (Brown and Brown, 1972; Pace, 1974; Frost and Bagnara, 1976). Adult male R. p. pipiens "have well developed internal vocal sacs but no external vocal sacs. The skin overlying the vocal sacs may be very stretched, especially during the breeding season, but this skin is not differentiated in any way from the surrounding skin" Pace, 1974). "The dorsal spots are usually much darker than the ground color and distinctly areolated." R. p. pipiens does not have a distinct tympanal light spot though an irregular blotch of lighter pigment may be present on the center of the tympanum (Pace, 1974). A complete supralabial stripe appears in R. p. pipiens and some other members of the complex but is absent from several other members (Frost and Bagnara, 1976).

B. Preliminary work using the semi-denervated frog brainstem

1. Surgical technique

The first surgical preparation used in this project from which neural correlates of electrically evoked mating calling activity were recorded, was the semi-denervated frog brainstem (Schmidt, 1974b). Frogs were hypothermally anesthetized by cooling them in ice water for about one hour. The anesthetized frogs were enucleated using iris scissors. The frogs were pinned, ventral side down, to a dissecting tray and covered with crushed ice. The skin over the dorsal surface of the skull was cut and removed. A dental drill with a burr bit was used to thin the skull over the brain. When the skull was sufficiently thin, a scalpel was used to make a small opening in the skull. That part of the skull lying over the brain was then cut and removed to expose the brain. The anterior three-fourths and the dorsal one-half of the posterior one-fourth of the cerebral hemispheres (Fig. 5) were removed to permit visualization of the anterior commissural ridge. This ridge served as a landmark for electrode placement following surgery. Next, the optic tectum, cerebellum, and medial parts of the inferior colliculi were removed. Removal of these structures is possible since they are not essential to the generation of normal release or mating

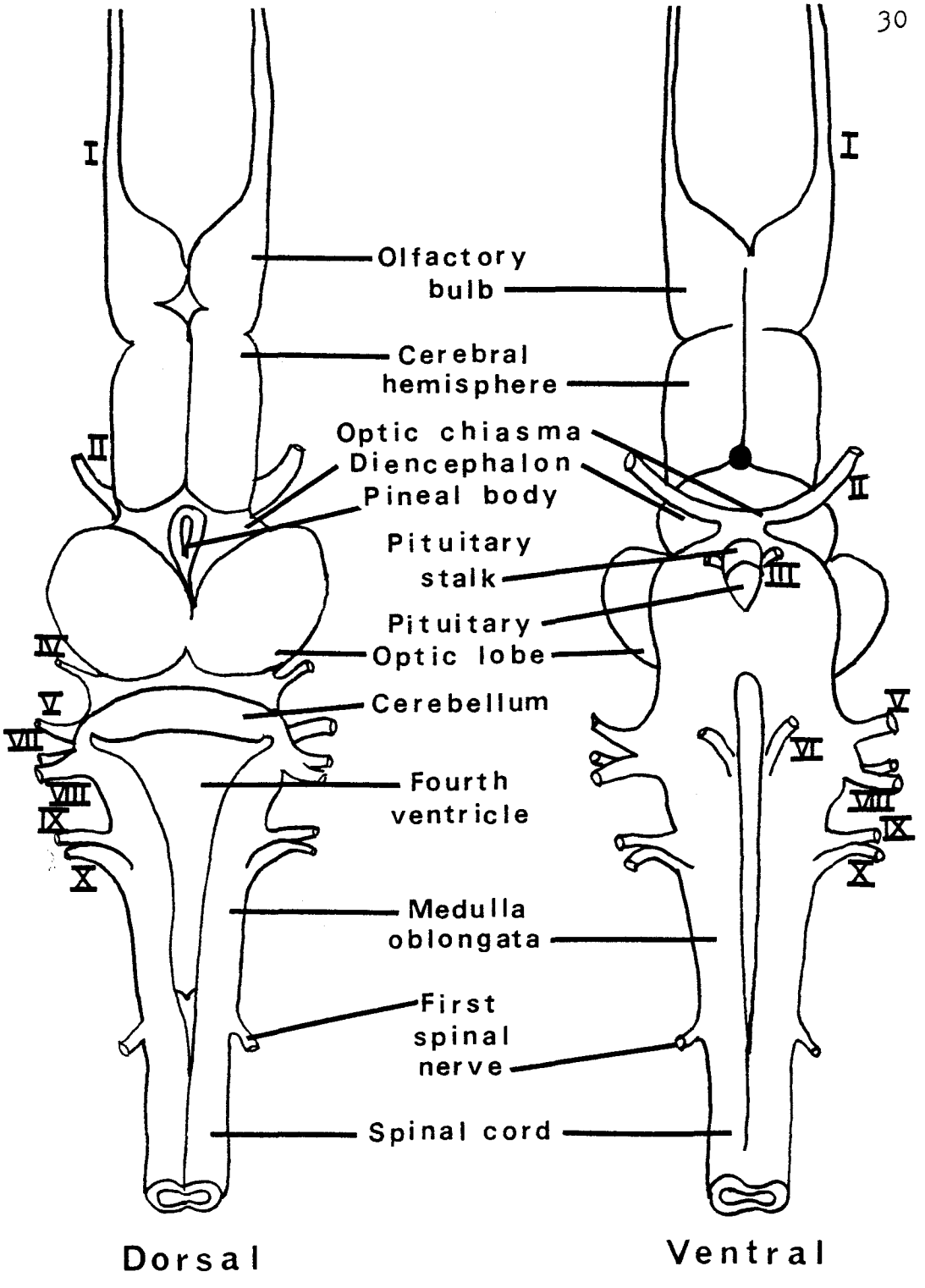

Fig. 5. Dorsal and ventral views of frog brain. Black solid midline circle marks site of electrical stimulation. (After Bloom, W. and Krekeler, C. 1964)

calling activity evoked by natural stimuli or by electrical stimulation of the brain (Schmidt, 1966, 1968a, 1971). Next, nerves were identified, isolated, and severed where appropriate. The sciatic nerves were cut through an incision along each side of the urostyle. The superior spinal vein was cut at the obex where it branches to each side of the medulla. The choroid plexus was cut at the midline. Each half of the choroid plexus was then retracted laterally and fused by electrocautery to the underlying muscles. The anterior branches of the superior spinal vein were left intact so that they continued to drain the medulla. The medulla was sectioned at the anterior edge of the brachial nerve. The hypoglossal nerves and cranial nerves V, VII, and IX were cut so as to minimize movement of the frog. The left vagus nerve (cranial nerve X) was cut while the right vagus was dissected free from the underlying tissue, preparing it for use in recording neural correlates. The long laryngeal nerve (a branch of the vagus) was cut close to where it leaves the vagus. The branch of the vagus that contains the short laryngeal nerve (that innervates only the glottal opener muscles of the larynx) was dissected free from the underlying tissue for a short distance from the divide and then cut. Either the long or short laryngeal nerve could be used for recording, but the short laryngeal was preferred. Because the only intrinsic laryngeal muscle innervated by

the short laryngeal nerve is the glottal opener muscle, this laryngeal nerve gave much sharper, more easily distinguishable recordings.

The dissecting tray was positioned in a micromanipulator stand. A screw hole drilled into the snout of the frog was used to attach the frog's head to a flat metal bar attached to the stand. The proximal end of the nerve was then sucked into a suction electrode. The suction electrode, made of stainless steel tubing, was held by wax to an L-shaped brass bar. The bar, in turn, was attached to the micromanipulator. The syringe used to form a suction was attached to the stainless steel tubing by a small diameter polyethylene tubing. A silver wire reference electrode was placed on a muscle mass near the short laryngeal nerve. The exposed brain was kept moist by occasional drops of frog saline placed directly onto the brain's dorsal surface.

2. Electrically evoked mating calling

Neural correlates of mating calling activity were evoked by midline stimulation of the anterior preoptic area through a bipolar stimulating electrode consisting of two 62 μm steel wires insulated to the tips. The tip of the stimulating electrode was placed on the midline at the anterior edge of the anterior commissural ridge. The electrode tip was lowered to an initial depth of 0.9 mm

into the brain and adjusted for maximum response, placing the tip in or near the anterior preoptic nucleus.

A Nuclear-Chicago constant current stimulator was used to deliver symmetrical biphasic pulses to the stimulating electrode at a rate of 70 Hz. Each phase of the stimulus was 0.5 msec and 400 - 800 μa.

The laryngeal nerve activity was picked up by the suction electrode, amplified, and displayed on an oscilloscope and recorded on a polygraph. The polygraph was modified so that the rapid electrical activity recorded from the laryngeal nerve was integrated and recorded as slow waves. Each wave cycle corresponded to a pulse of activity occurring in the laryngeal nerve. The polygraph was later replaced by a Honeywell 1508B Visicorder (a light-beam, direct-recording oscillograph) capable of recording the rapid activity occurring in the nerve up to a direct current bandwidth of 3,300 Hz without the necessity of integrating it first.

3. Disadvantages of the semi-denervated frog brainstem preparation

The semi-denervated brainstem had several disadvantages. The procedure was difficult to do. The viability percentage was low. These two disadvantages were due to a host of factors. The procedure depended on visual placement of the stimulating electrode. In order to do

this accurately, certain areas of the brain which obstructed the view of pertinent anatomical landmarks had to be removed. The cutting and removing of neural tissue often resulted in bleeding and seepage of blood into the visual field, making accurate placement of the stimulating electrode difficult. In order to keep the preparation viable, an adequate blood supply to the brain had to be retained. The placement of electrodes was restricted by the necessity of avoiding damage to the blood supply already compromised by the necessary surgery.

Another problem with the semi-denervated brainstem technique resulted from the use of a suction electrode to record from the branch of the short laryngeal nerve. The nerve had to be sucked into the electrode. This resulted in a portion of the nerve being exposed to the air on all sides. This caused drying of the nerve which could alter recordings. In order to keep the nerve moist, saline was dropped frequently onto the exposed nerve, but this method was cumbersome.

An additional problem occurred whenever drugs were to be administered to the preparation. There was no good way to apply known quantities of the drug directly to the brain's calling circuits. Drug solutions applied directly to the exposed portions of the brain could be picked up by the vasculature of those sections of the brain into which the drug solution diffused. Thus, the distribution of the

drug to the brain was uneven. Drugs could reach the peripheral system and cause peripheral drug effects that complicate interpretation of central drug effects.

The difficulties encountered with the semi-denervated brainstem preparation led to its abandonment in favor of the completely isolated frog brainstem preparation (Schmidt, 1976).

C. Isolated frog brainstem technique

1. Introduction

Because of limitations and disadvantages of the semi-denervated frog brainstem preparation, Schmidt developed the technique for studying neural correlates of calling in the isolated frog brainstem (Schmidt, 1976). Modifications and adaptations were necessary for this technique to be used in drug studies. To allow drugs to flow rapidly into the bath and to be rapidly flushed from the bath, the original tissue bath was redesigned.

2. Advantages of the isolated brainstem over the semi-denervated brainstem

The isolated brainstem preparation has several advantages over the semi-denervated brainstem preparation. Bleeding is not a problem in the isolated brainstem. This means that blood does not obscure the view for electrode placement and that the integrity of vessels in the brain

does not have to be preserved. The specimen will not move of its own accord, dislodging electrodes. The technique eliminates any possible peripheral neural or hormonal feedback. The surgical procedure was easier than the denervated brainstem technique. The preparations seemed to be more viable than the semi-denervated brainstem preparations (Schmidt, 1976). In addition, the uniqueness of the isolated brainstem preparation made it advantageous for drug studies. Drugs could be applied directly to the brain without having to pass the blood brain barrier. Any drug effect was due to the effect on the central nervous system since all possible peripheral drug effects were eliminated.

3. The tissue bath

The first published account of the isolated brainstem technique (Schmidt, 1976) described the construction of a tissue bath built for the isolated frog brainstem. The original tissue bath worked well for recording neural correlates of mating calling activity but did not provide a means of rapidly introducing or removing different oxygenated saline solutions. In order to do any type of drug study, a method for rapidly introducing and rapidly removing a drug dissolved in oxygenated saline was needed.

A new tissue bath for maintaining the isolated brainstem was designed (Fig. 6) to meet the following

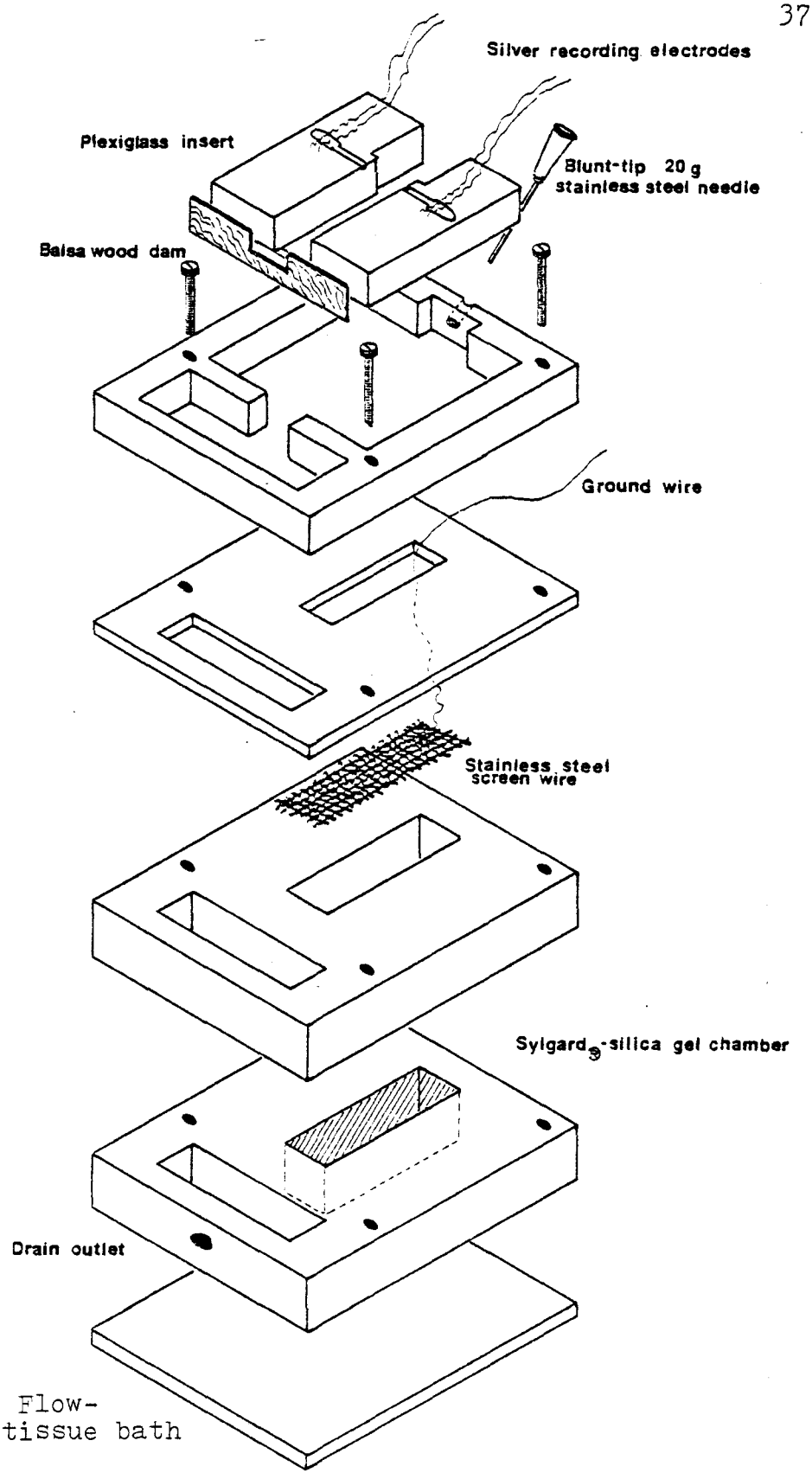

Fig. 6. Flow-through tissue bath

requirements: 1) allow for continuous superfusion of all sides of the isolated brainstem with oxygenated saline, 2) provide room for performing surgical procedures on the submerged isolated brainstem, 3) provide for the rapid introduction and removal of known concentrations of drugs dissolved in oxygenated saline, 4) provide recording electrodes for the laryngeal nerves, and 5) allow an adequate view of the brain for the placement of stimulating and recording electrodes.

The plexiglass tissue bath that was designed and constructed for this project contains a stainless steel screen on which the brainstem rests. The isolated brainstem is positioned on the screen with insect pins that anchor into transparent encapsulating resin (Sylgard 184-Dow Corning) located in the chamber just below the screen. Oxygenated saline enters one end of the chamber below the screen and flows upward through the screen and around the brainstem. The saline flows out the opposite end of the bath over a balsa wood dam at a height sufficient to keep the brainstem completely submerged in saline. The saline is oxygenated in leveling bulbs before entering the bath. Having two to three leveling bulbs attached to the apparatus enables an easy conversion from one saline solution to another. Thus, drugs dissolved in oxygenated saline can be introduced easily into the bath and subsequently washed out of the bath.

The bath is fitted with a plexiglass insert to provide recording electrodes for the laryngeal nerves. It has a trough on each side that opens to the inner chamber of the bath through a small groove. Each trough has two silver recording electrodes.

4. Surgical and stimulating technique

The surgical procedure for preparing the isolated brainstem as first described by Schmidt in 1976 and as used in this project is summarized as follows:

The frog is hypothermally anesthetized in ice water for at least one hour. It is decapitated with scissors by cutting through the commissures of the mouth and then it is enucleated. The head is pinned with its ventral side up onto an iced dissecting tray. Keeping the head chilled in this way helps preserve the brain's viability. The vertebrae leading to the skull are cut on each side with iris scissors to expose the spinal cord. Two ventrolateral cuts are made through the foramen magnum and the incisions are continued anteriorly past the cerebrum to an area directly over the olfactory bulbs (Fig. 5). The resulting tissue flap is removed. The vagus nerves are identified because, as with the semi-denervated preparation, the short laryngeal nerve (a branch of the vagus) is to be used for recording neural correlates. Each vagus nerve passes from the brain through a foramen.

These foramina must be cut and the vagus and short laryngeal nerves then freed from surrounding connective tissue. Short portions of the brachial nerves are also dissected free and left attached to the brain. The brain from the olfactory bulbs to a level just posterior to the brachial nerves and including its attached vagus and brachial nerves, is removed from the skull.

This isolated brainstem is then placed ventral side down on the screen in the bath. It is secured in place with insect pins through each cerebral hemisphere and between the roots of the brachial nerves on each side.

The tissue bath already has a cold saline solution running through it. The saline is oxygenated in the leveling bulbs with a 95% O_2, 5% CO_2 gas mixture. The saline solution used is one described by Tebēcis and Phillis (1969) consisting of 100 mM NaCl, 2.5 mM KCl, 2.5 mM Na_2HPO_4, 0.45 mM NaH_2PO_4, 1.9 mM $CaCl_2$, 12 mM $NaHCO_3$, and 2.8 mM d-glucose. The saline is cooled in the tubing as it passes through an ice water bath before entering the tissue bath.

Once the brainstem is secured in the bath, further surgery is performed. The epithalamus is split down the midline. The cerebellum, choroid plexus, dorsal parts of the optic tectum, and medial parts of the inferior colliculus (areas not essential for calling - Schmidt, 1973) are removed, exposing the floor of the ventricles where

electrodes will be placed.

Further surgery varies with the desired approach for electrode placement. The brainstem may be left in place with the dorsal side up for electrode placement or it may be unpinned, turned over, and repinned to the screen with the ventral side up (Fig. 5). The first approach for placement of electrodes is called the dorsal approach since it is the dorsal surface that is viewed through the Zeiss operating microscope and it is the dorsal surface into which the electrodes are placed. The latter approach is called the ventral approach since the ventral surface is viewed and it is the ventral surface into which the electrodes are placed.

a. Dorsal approach to isolated brainstem

The next surgical step in using the dorsal approach is to place two additional pins through the preparation, one through each lateral margin of the optic tectum. The plexiglass insert is then placed into the tissue bath. The short laryngeal nerve on each side is then placed through a slit in the insert and into its respective groove. The nerve is placed over the two silver recording electrodes in each groove. The slit opening into each groove is sealed with white petroleum jelly. Mineral oil is placed into each groove (or trough) covering the nerve and recording electrodes in order to prevent

the nerves from drying and to help isolate the nerves electrically.

Once surgery is completed, the bath is placed into the micromanipulator stand. Now the saline flowing through the bath is allowed to gradually warm to 20.5°C while the bipolar stimulating electrode is placed. The stimulating electrode is placed into the diencephalon using Narishige micromanipulators. It is placed on the midline just anterior to the anterior commissural ridge and lowered to an approximate depth of 0.8 to 0.9 mm. A Nuclear-Chicago constant current stimulator is used to generate symmetrical biphasic pulses. Each pulse of the stimulus is 0.5 msec, 70 Hz, and 500 to 700 μA.

b. Ventral approach to isolated brainstem

In the ventral approach, the brainstem surgery is the same as for the dorsal approach until the insert is ready to be placed into the tissue bath. At this stage, the pins are removed from the brainstem, the brainstem is turned over, and the brainstem is repinned to the screen with the ventral surface upward. The pins are placed through the same structures as in the dorsal approach (i.e. the cerebral hemispheres, lateral optic tectum, and between the dorsal and ventral roots of the brachial plexus) except that now the pins enter the ventral surface and pass out the dorsal surface.

Next, as with the dorsal approach, the plexiglass insert is placed into the bath and the short laryngeal nerves are placed through the slit-like opening into the troughs (or grooves) and over the two silver recording electrodes on each side. The slit openings are sealed with petroleum jelly and the troughs are filled with mineral oil to cover the short laryngeal nerves. The bath is then placed into the micromanipulator stand. As with the dorsal approach, the saline is now allowed to gradually warm. The Narishige micromanipulator is used to place a concentric stimulating electrode on the ventral surface of the anterior preoptic area. The concentric electrode (designed and built by R. S. Schmidt) is made of a Teflon-coated 250 μm silver wire core in a 22 gauge stainless steel tube insulated to the tip with heat-shrinkable Teflon tubing. The core extends about 500 μm beyond the tube. Only the tip of the core is placed in contact with the ventral surface of the anterior preoptic area. A Nuclear-Chicago constant current stimulator is used to deliver biphasic pulses at 70 Hz, 400 to 500 μa with each pulse lasting 0.5 msec. The core of the concentric electrode is always attached to the negative terminal of the stimulator.

c. Advantages and disadvantages of dorsal and ventral approach

The dorsal approach was preferred when it was

desired to place electrodes into the medullary calling circuits. It provided a view of the dorsal surface of the medulla and the floor of the fourth ventricle, thus providing visual landmarks for the placement of recording or stimulating electrodes into the medullary calling circuits. Although the medullary calling circuits could be reached by the ventral approach, the ventral surface provided fewer anatomical landmarks, making the accurate placement of electrodes more difficult. The dorsal approach had the advantage of eliminating the time required to unpin, turn over, and repin the brainstem.

A major disadvantage of the dorsal approach was the difficulty of accurately placing a stimulating electrode into the anterior preoptic nucleus. The anterior preoptic nucleus lies on the ventral surface of the diencephalon. Therefore, the stimulating electrode must pass through about 0.8 to 0.9 mm of tissue in the diencephalon before entering this area. With this much brain tissue to penetrate and with the inability to visualize the target directly, accurate placement was hindered. Also, the size of the diameter of the electrode that could be used was limited. In this research project, only the bipolar stimulating electrode could be used in the dorsal approach.

The ventral approach had the advantage of visualization of the anterior preoptic area and use of the concentric electrode. Limited comparisons of the concen-

tric and bipolar electrodes suggested that calling could be elicited more consistently and for a longer period of time with the concentric electrode (Schmidt, 1976).

The ventral approach was undesirable, however, when placing electrodes into the medullary calling circuits. Accurate placement was more difficult because there are fewer landmarks for placement on the ventral surface than there are on the dorsal surface.

5. Temperature control

The temperature of the oxygenated saline in the tissue bath was controlled by heating or cooling the saline while in polyethylene tubing leading to the bath. The tubing was coiled and placed in a water bath. The water in this bath holding the tubing was heated by placing an incandescent lamp just above the water. The water was cooled by adding crushed ice to the bath in small increments. The temperature of the saline in the tissue bath was measured with a mercury thermometer calibrated in units of 0.2 C^{o}.

This temperature control method was used in Chapters III and V to maintain a tissue bath temperature of 20.5^{o}C. Changes in temperature were accomplished with this method for the experiments in Chapter IV.

6. Recordings of neural correlates

Neural correlates of mating calling were recorded following electrical stimulation of the anterior preoptic nucleus. The recordings were made from the short laryngeal nerve or from the area of the pretrigeminal nucleus in the medulla. The silver electrodes in the troughs of the plexiglass insert of the tissue bath recorded activity in the short laryngeal nerves while bipolar electrodes inserted into the pretrigeminal area recorded activity from this area of the medulla. Regardless of the area used, the activity was amplified and recorded on a Honeywell 1508B Visicorder light-beam oscillograph. Recordings from the short laryngeal nerves were made at an amplifier bandwidth of 3 - 3000 Hz. Recordings from the pretrigeminal area were made at a bandwidth of 3 - 100 Hz. The amplifier output was monitored by an oscilloscope and an audiomonitor. A simplified circuit diagram of the equipment used appears in Fig. 7.

The neural correlates of mating calling activity recorded from the short laryngeal nerves (Fig. 8) were characterized as consistently having pulses of electrical activity (the vocal phase) followed by a tonic burst of activity at the end of the call (the inspiratory phase).

Recordings from the pretrigeminal nucleus (Fig. 8) on the other hand, showed slow waves during the vocal

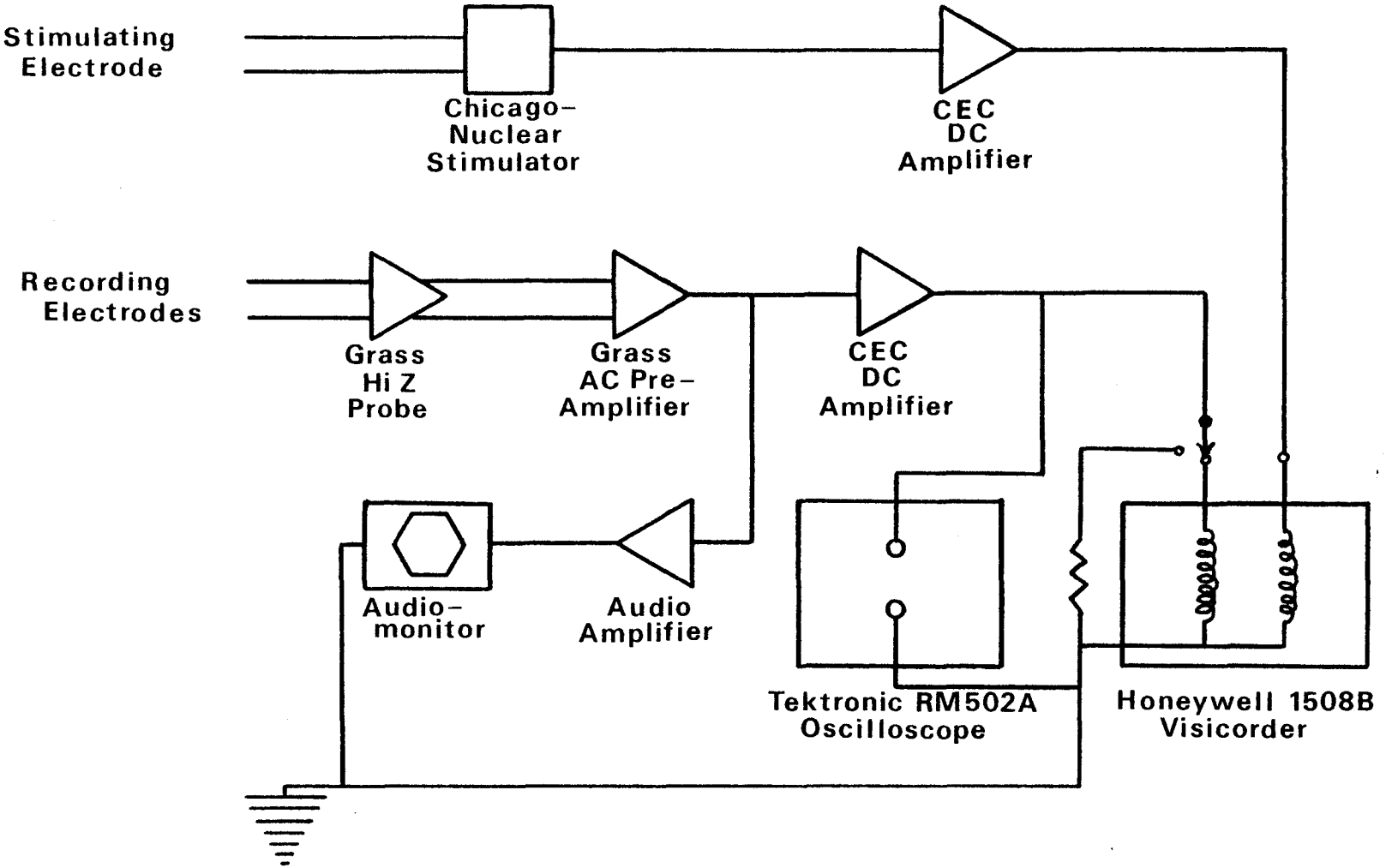

Fig. 7. Wiring diagram of stimulating and recording equipment. Only one of four channels for recording neural correlates is shown.

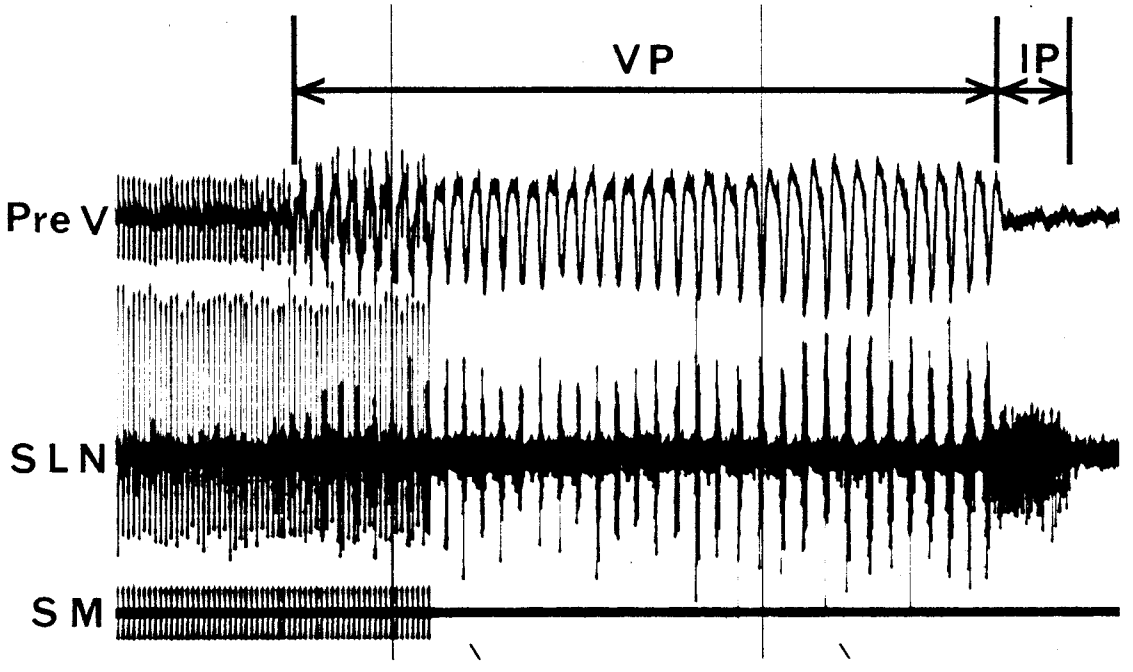

Fig. 8. Neural correlates of electrically evoked mating calling activity. Pre V = recording from region of the pretrigeminal nucleus, SLN = recording from short laryngeal nerve, SM = recording from stimulus monitor, VP = vocal phase, IP = inspiratory phase. Vertical time lines occur at one second intervals.

phase. Each slow wave recorded corresponded to a pulse of activity recorded from the short laryngeal nerve. The tonic inspiratory phase usually was not detected in recordings at the amplifier bandwidth of 3 - 100 Hz used for the pretrigeminal nucleus.

The recordings from these two areas are correlated with mating calling in the intact frog. Each pulse of the vocal phase (VP) corresponds with the brief opening of the glottis and a sound pulse of the mating call. The vocal phase evolved from the expiratory phase of pulmonary respiration. The short, terminal burst of laryngeal nerve activity during the inspiratory phase (IP) corresponds with the tonic opening of the glottis and the return of air to the lungs as occurs in the inspiratory phase of breathing (Schmidt, 1976).

Permanent records were made with the Honeywell 1508B Visicorder light-beam oscillograph onto Kodak Linagraph Direct Print Paper Type 1895 at a paper speed of 50 mm/sec. The oscillograph was set to record the neural correlates on four different channels (Fig. 7). A fifth channel was used to monitor the stimulus. Seldom were all five channels used at once.

Whenever possible, recordings were made from both short laryngeal nerves even though both short laryngeal nerves showed the same activity. By recording from both, when the quality of the recording was better from one

nerve than from the other, the best recording was selected.

Usually, when recordings were made for the region of the pretrigeminal nuclei, only one side was recorded because of the difficulty in placing two recording electrodes into the small area of the medulla containing the pretrigeminal nuclei, because of the additional trauma to the medullary circuits caused by inserting two recording electrodes into the pretrigeminal area, and because pretrigeminal area recordings made from each side of the brain would show the same activity since the activity of both sides is synchronized.

D. Quantitation of data

Quantitation of the recordings enabled several variables of the neural correlates of frog calling to be analyzed and described. Those variables analyzed were initial pulse rate, vocal phase pulse rate, duration of the vocal phase, total number of pulses in a vocal phase, and stimulus-response latency.

To facilitate quantitation, a ruler was made using Kodak Linagraph^{R} paper. The Visicorder was run at a paper speed of 50 mm/sec. Vertical time bars were set to appear on the Linagraph paper every 0.1 seconds. Every tenth time bar was heavier and darker, indicating the completion of a second. A strip of this paper on which only the time bars appeared was folded in half lengthwise and marked.

The first heavy time bar line on the left was marked as zero (0). Each one second time bar after that was numbered successively 1, 2, 3, etc. The half-second time bars were darkened with a fine point black pen. This ruler was then ready to be used.

The pulses were measured in one second segments by placing the zero mark of the ruler at the start of the first pulse of activity and counting all the pulses between each one second mark. If the end of the pulsing, i.e. the end of the vocal phase, occurred between one second marks, then the pulses from the last complete one second segment of the vocal phase were counted and the one-tenth second time bars were used to determine the amount of additional time to complete the vocal phase. Counting the pulses occurring during successive one second segments of the vocal phase showed the amount of change from one second to the next.

Using this method of counting pulses, the initial pulse rate (initial one second pulse rate) was determined by counting the number of pulses that occurred during the initial one second segment of the vocal phase.

The vocal phase pulse rate was determined by dividing the total number of pulses per vocal phase by the vocal phase duration.

Vocal phase duration was measured by placing the ruler at the beginning of the first pulse of activity in

the vocal phase and measuring to the end of the last pulse.

The total number of pulses in a vocal phase was determined by counting each pulse recorded during the vocal phase of calling.

Stimulus-response latency measured the time between the beginning of the stimulus monitor recording and the start of the first pulse.

E. Criteria for acceptance

1. Isolated brainstem preparation

An isolated brainstem preparation was considered acceptable for studies of mating calling activity only if neural correlates were consistently evoked by electrical stimulation of the anterior preoptic nucleus and if the preparation remained viable for the duration of the experiment. Each preparation had to show mating calling activity after electrical stimulation by symmetrical biphasic pulses of 0.5 msec duration and a maximum of 800 μa of current delivered at a rate of 70 Hz for a maximum of seven seconds.

2. Recordings of neural correlates

An acceptable short laryngeal nerve recording of neural correlates of electrically evoked mating calling

activity (Fig. 2 and 8):

1) clearly showed a pulsed vocal phase followed by a tonic inspiratory phase,
2) had a vocal phase lasting at least one second,
3) had a vocal phase with a clearly discernible beginning and a clearly discernible end, thus permitting quantitation of the variables being studied,
4) had pulses that occurred in an orderly, not erratic, sequence from the start to the end of the vocal phase.

An acceptable recording from the region of the pretrigeminal nucleus in the medulla (Fig. 8):

1) had a vocal phase consisting of slow waves,
2) did not necessarily have a visible inspiratory phase,
3) had a vocal phase lasting at least one second,
4) had a vocal phase with a clearly discernible beginning and a clearly discernible end,
5) had slow waves that occurred in an orderly sequence from the start to the end of the vocal phase.

CHAPTER III

DESCRIPTION OF NORMAL NEURAL CORRELATES

A. Introduction

Neural correlates of electrically evoked mating calling activity in the isolated frog brainstem have been described qualitatively for adult (Schmidt, 1976) and juvenile frogs (Schmidt, 1978); however, neural correlates have not been described quantitatively. A quantitative description of the neural correlates of electrically evoked mating calling activity was made by compiling and quantitating the data from 42 isolated brainstem preparations.

B. Methods

1. Experimental design

The experiments were done in 1978 on adult male Rana pipiens pipiens purchased from Hazen, Inc., Vermont. The ventral surgical approach was used. The temperature for each preparation was controlled at $20.5^{\circ}C \pm 0.2^{\circ}C$. Each preparation was electrically stimulated in the preoptic area seven times at two-minute intervals. Five different variables were selected to describe the neural correlates of mating calling activity. These were initial

pulse rate, vocal phase pulse rate, vocal phase duration, total pulses per vocal phase, and stimulus-response latency. The averages were calculated for each individual preparation. The results of the 42 preparations were combined.

With the quantitated data, it was then possible to compare the neural correlates of frog calling with actual frog calling patterns reported in the literature to verify that the recordings were indeed correlated with mating calling patterns in the intact frog.

2. Statistics

a. Measures of variability

Variability of neural correlates of mating calling recorded from the different brainstem preparations was statistically evaluated by standard deviation, coefficient of variation, and skewness. These were calculated by:

$$\text{Standard deviation } (S) = \sqrt{\frac{\Sigma(X - \bar{X})^2}{n - 1}}$$

$$\text{Coefficient of variation } (C.V.) = \frac{S}{\bar{X}} \times 100\%$$

$$\text{Skewness} = \frac{3 \cdot (\bar{X} - Md)}{S}$$

where X = value of variable

$\bar{X}$ = mean of X values

Md = median

n = number of experiments

b. The difference method test of significance

The difference between the pulse rate of different one-second segments of the same vocal phase were tested for significance using the following formulas and a table of the critical values of the t statistics:

$$S_{X_D} = \sqrt{\frac{\Sigma(X_D - \bar{X}_D)^2}{n - 1}}$$

$$SE_{\bar{X}_D} = \frac{S_{X_D}}{\sqrt{n}}$$

$$t = \frac{\bar{X}_D}{SE_{\bar{X}_D}}$$

where X_D = difference between pulse rates of paired one-second vocal phase segments of a preparation

$\bar{X}_D$ = mean of all the differences (X_D)

S_{X_D} = standard deviation of the difference

$SE_{\bar{X}_D}$ = standard error of the mean difference

n = number of pairs (i.e. number of isolated brainstem preparations)

C. Results

1. Rate of decline in pulse rate

In general, the pulse rate of recordings of neural

correlates decreased as the vocal phase progressed (Fig. 9). The number of pulses were counted during each second-long segment of the vocal phase to determine this pattern. Of the 42 preparations, 18 had vocal phases lasting less than two seconds in at least four of their seven recordings of mating calling activity. The remaining 24 preparations had vocal phases lasting two seconds or longer in at least four of their seven recordings of mating calling activity. All 24 preparations had a faster mean pulse rate during the initial one-second segment of the vocal phase than during the following one-second segment. The mean pulse rate for the initial second of all 24 preparations was 21.5 pulses per second. The mean pulse rate for the next second was 18.7 pulses per second. The difference in mean pulse rate was statistically significant at the 0.001 level.

Six of the 42 preparations had vocal phases lasting three or more seconds in at least four of their seven recordings of mating calling activity. The mean pulse rate for each one-second segment of the six preparations showed a decreasing pulse rate as follows:

Initial second = 21.1 pulses/second

Second second = 18.4 pulses/ second

Third second = 18.0 pulses/second.

The difference between the initial one-second segment and the second segment of the vocal phases of the six

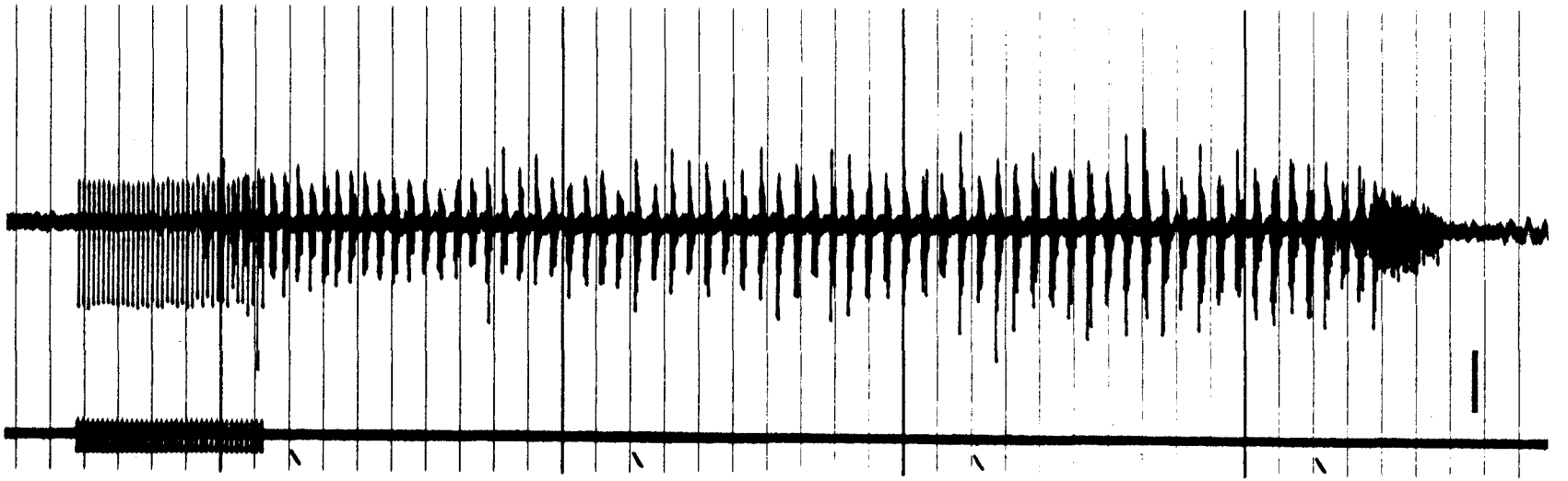

Fig. 9. Neural correlates of mating calling activity showing a decrease in pulse rate as the vocal phase progressed from left to right. Upper trace showed a pulsed vocal phase followed by a tonic inspiratory phase as recorded from the right short laryngeal nerve of the isolated frog brainstem. The pulse rate was fastest at the beginning of the vocal phase. Lower trace was recorded from stimulus monitor. Vertical time bars occurred every 100 msec with a bolder line occurring every one second. Amplitude scale (lower right) = 100 μV.

preparations was statistically significant at the 0.05 level, but the difference between the second one-second and the third one-second segment was not statistically significant. In two of these six preparations, the pulse rate was greater in the third second than in the second second. The pulse rates for the first preparation were:

Initial second = 19.4 pulses/second

Second second = 18.3 pulses/second

Third second = 18.6 pulses/second.

The pulse rates for the second preparation were:

Initial second = 19.7 pulses/second

Second second = 16.9 pulses/second

Third second = 17.0 pulses/second.

Two preparations had recordings lasting longer than four seconds. Their pulse rates decreased during each subsequent second of the vocal phase. The pulse rate of one of these preparations was:

Initial second = 23.0 pulses/second

Second second = 19.6 pulses/second

Third second = 18.5 pulses/second

Fourth second = 18.0 pulses/second.

2. Initial pulse rate

The statistical values for the initial pulse rate of neural correlates of mating calling activity of the 42 preparations (n = 42) were:

Range.....17.4 to 26.9 pulses/second

Median.....20.95 pulses/second

Mean.....21.56 pulses/second

Skewness.....0.78

Standard deviation.....2.35 pulses/second

Coefficient of variation...10.90%

The data obtained from all 42 preparations were grouped into classes with a class width of 1.0 pulses/second. The grouped data was presented in a histogram (Fig. 10).

3. Vocal phase pulse rate

The vocal phase pulse rate was calculated so that pulse rates of neural correlates from the isolated brainstem could be compared with call pulse rates reported in the literature for intact frogs. Both are calculated in a similar manner.

Statistically, the mean pulse rate of the 42 preparations (n = 42) was:

Range.....16.2 to 24.8 pulses/second

Median.....19.60 pulses/second

Mean.....20.01 pulses/second

Skewness.....0.63

Standard deviation.....1.96 pulses/second

Coefficient of variation...9.80%

The data were grouped into classes with a class width of one pulse per second. The histogram in Fig. 11 showed

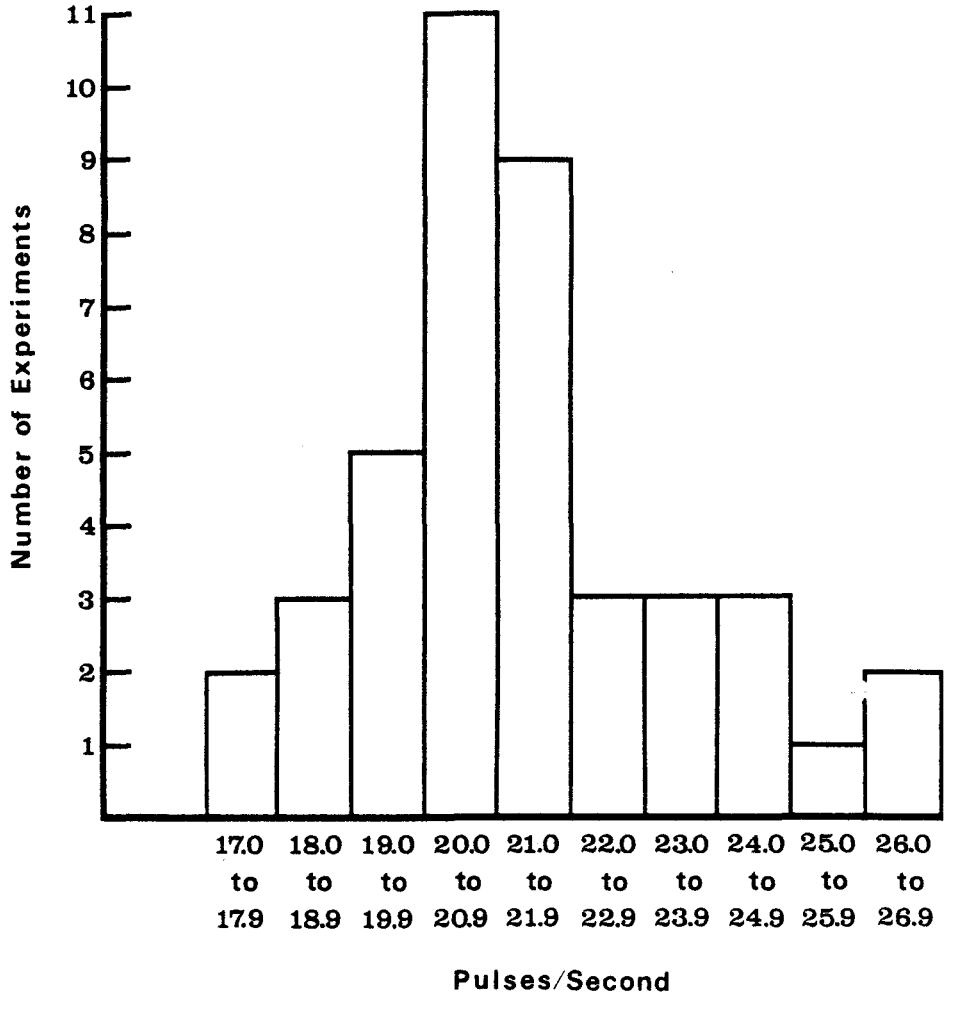

Fig. 10. Distribution of initial pulse rates from 42 isolated frog brainstem preparations.

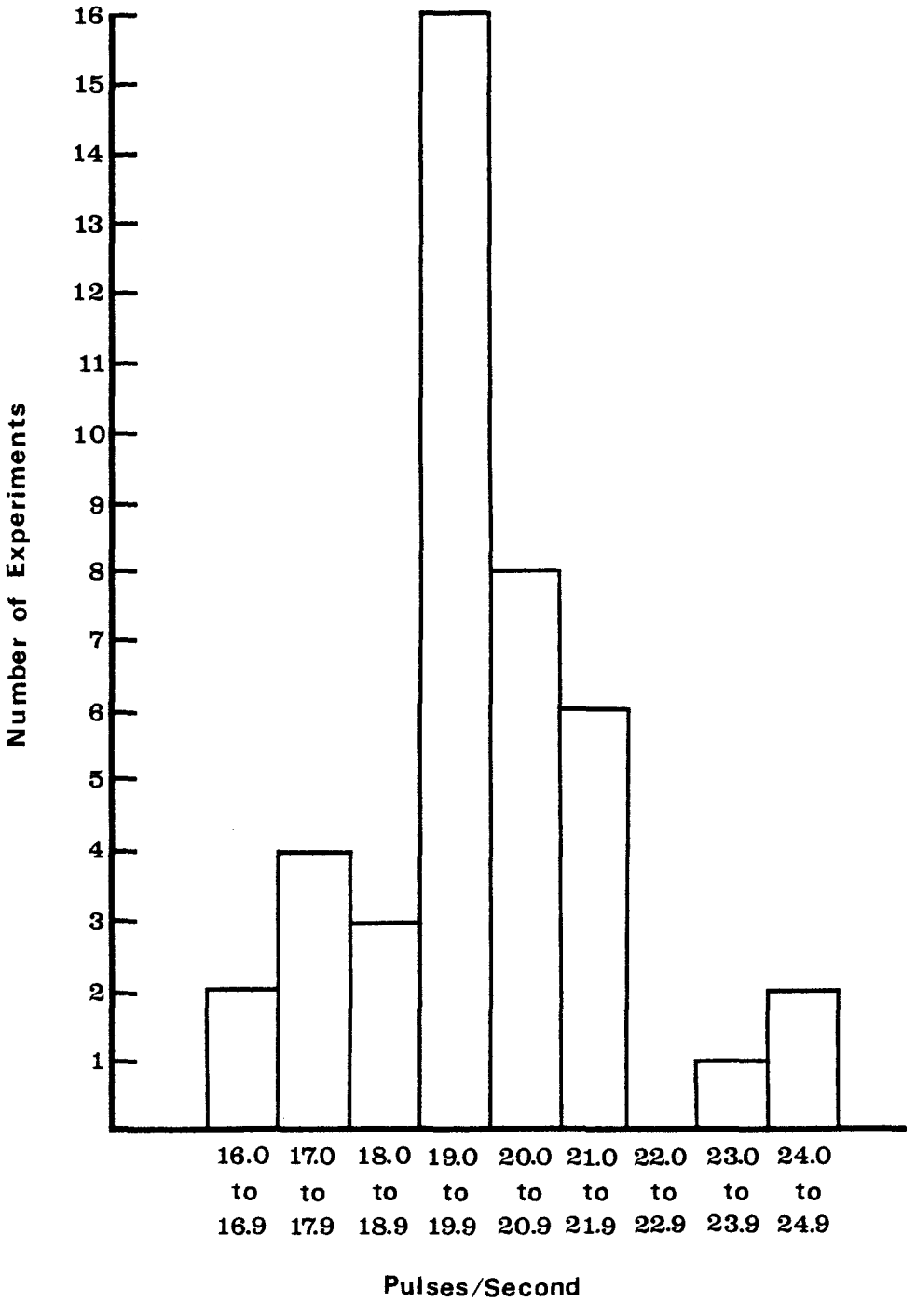

Fig. 11. Distribution of vocal phase pulse rates from 42 isolated frog brainstem preparations.

the grouped data.

4. Vocal phase duration

The vocal phase duration (n = 42) was described as:

Range.....1.26 to 5.79 seconds
Median.....2.08 seconds
Mean.....2.35 seconds
Skewness.....0.90
Standard deviation.....0.90 seconds
Coefficient of variation...38.3%

The data were grouped into classes with a class width of 0.40 seconds. The histogram in Fig. 12 showed the grouped data.

5. Total pulses per vocal phase

The calculations of the total pulses per vocal phase (n = 42) were combined to give the following values:

Range.....19.9 to 110.6 pulses
Median.....41.6 pulses
Mean.....46.7 pulses
Skewness.....0.92
Standard deviation.....16.7 pulses
Coefficient of variation...35.8%

The data were grouped into classes with a class width of 5.0 pulses. The histogram in Fig. 13 showed the grouped data.

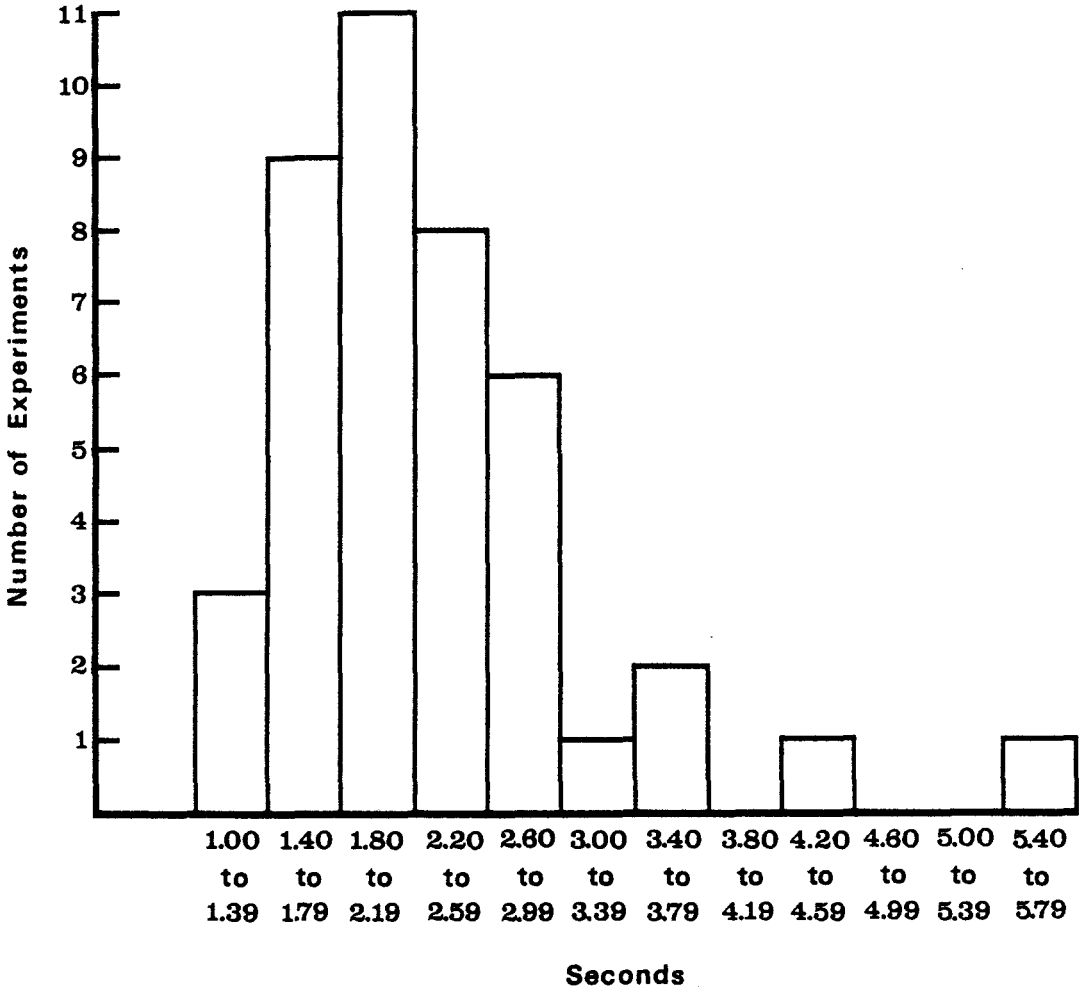

Fig. 12. Distribution of vocal phase durations from 42 isolated frog brainstem preparations.

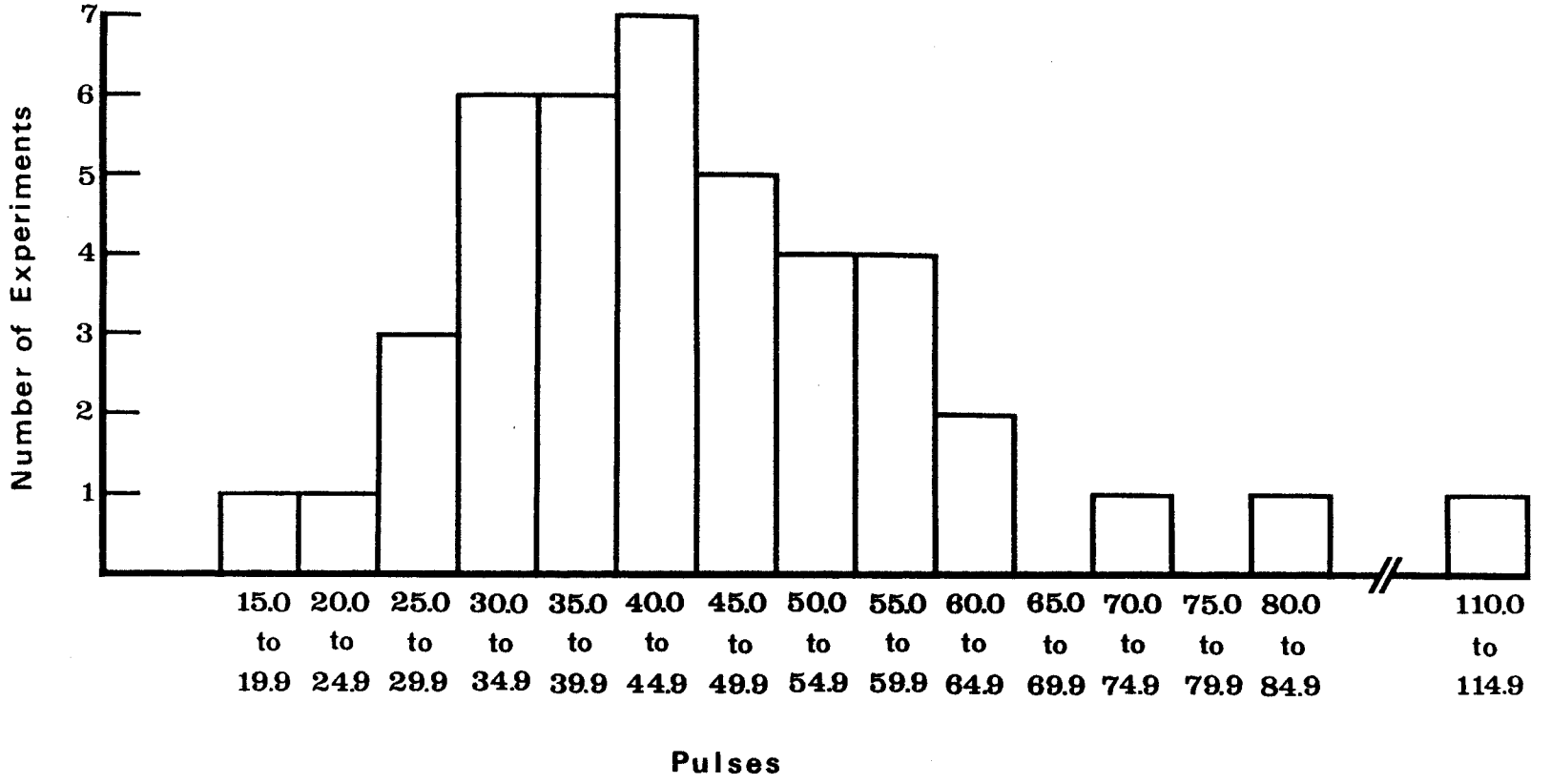

Fig. 13. Distribution of total pulses per vocal phase from 42 isolated frog brainstem preparations.

6. Stimulus-response latency

The stimulus-response latency data (n = 42) were pooled to give the following statistical information:

Range.....0.21 to 3.47 seconds

Median.....0.77 seconds

Mean.....1.14 seconds

Skewness.....1.32

Standard deviation.....0.84 seconds

Coefficient of variation...73.7%

The data were grouped into classes with a class width of 0.3 seconds. The histogram in Fig. 14 showed the grouped data.

D. Discussion

1. Characteristics of normal neural correlates

The histograms were used to describe the frequency distributions of the 42 brainstem preparations that were quantitated. The data from four of the five variables studied yielded histograms (Fig. 10, 11, 12, and 13) that showed a relatively normal distribution around the mean. The initial pulse rate (Fig. 10) and the vocal phase pulse rate (Fig. 11) resulted in histograms with the density of measurements greatest in the center and diminishing to the left and the right. The vocal phase pulse rate data had a mean only slightly larger than its median, i. e. it was

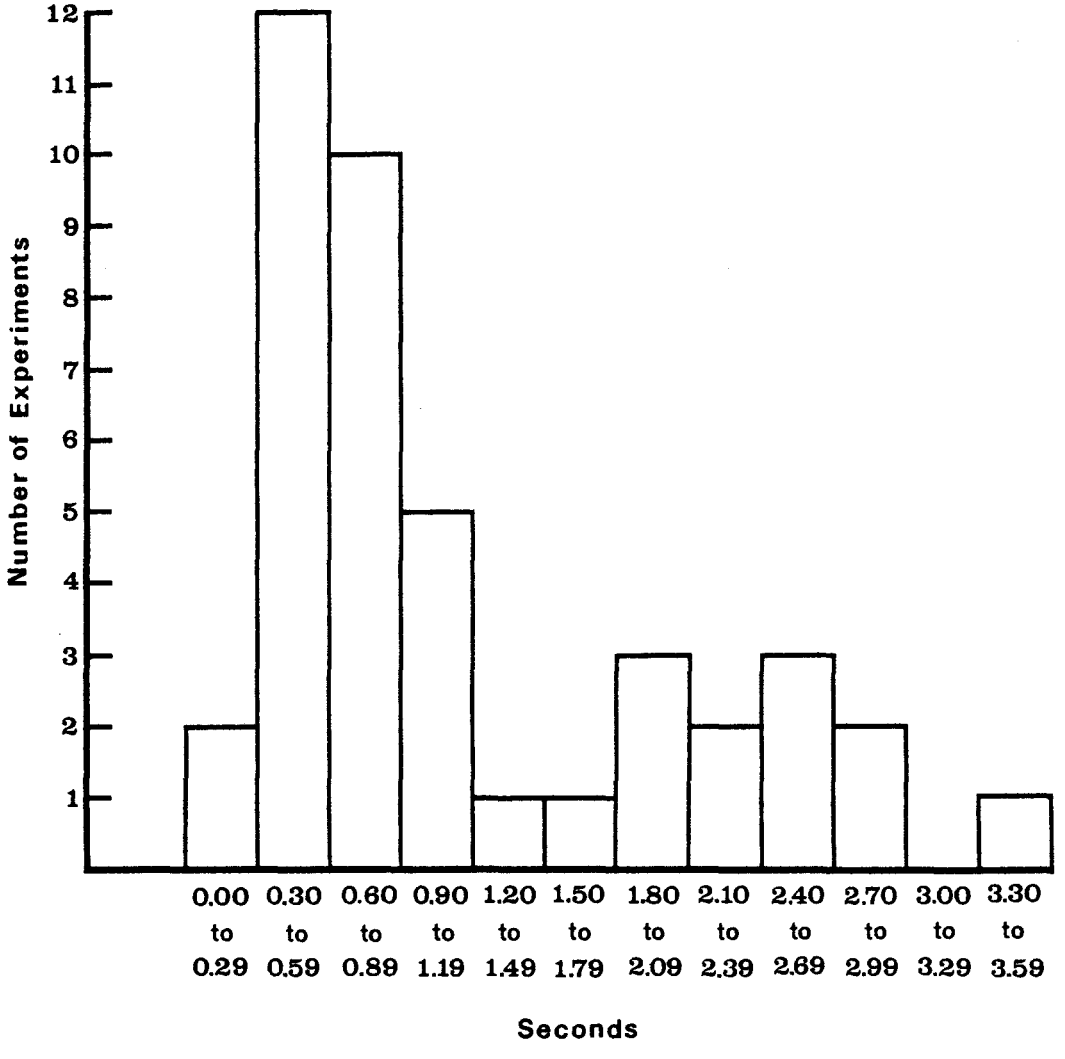

Fig. 14. Distribution of stimulus-response latencies from 42 isolated frog brainstem preparations.

slightly skewed to the right. The initial pulse rate also had a mean slightly larger than its median, resulting in a slightly greater degree of skewness to the right than occurred in the vocal phase pulse rate.

The histograms for the vocal phase duration (Fig. 12) and the total pulses per vocal phase (Fig. 13) were skewed to the right. The obvious tail to the right in the histograms illustrated this skewing. The rest of the data in both histograms were relatively evenly distributed about the mean.

Unlike the other four histograms which were easily interpreted, interpretation of the histogram for stimulus-response latency (Fig. 14) was debatable. It could be considered as a unimodal distribution with a skew to the right or as a bimodal distribution. The unimodal interpretation seemed more logical because the latency values could not go to zero, i.e. some stimulation time was always required for triggering mating calling activity. This meant that the values could be skewed only to the right. Also, only two classes fell to the left of the median. Nevertheless, whether a larger sampling would have resulted in a more normally distributed histogram has not been shown.

2. Coefficient of variation

Coefficients of variation were used to measure the

variability among the 42 preparations. By definition a coefficient of variation is a relative measure of variability in which the standard deviation is expressed as a per cent of the mean. For the data accumulated in this research project, coefficient of variation provided a meaningful method for determining which of the five parameters were least variable. The initial pulse rate (10.9%) and the vocal phase pulse rate (9.8%) showed the least variability of the five variables within the 42 preparations studied. This was expected since pulse rates are important in call recognition (Loftus-Hill and Littlejohn, 1971). The coefficients of variation for the vocal phase duration (38.3%) and the total pulses per vocal phase (35.8%) each were more than three times greater than the coefficients of variation of the pulse rates. The stimulus-response latency had the greatest variability with a coefficient of variation of 73.7%.

3. Comparison of neural correlates with mating calls

Description of mating call characteristics of members of the Rana pipiens complex living in the central United States and in Illinois provided a basis for comparison of neural correlates of mating calling with mating call patterns observed in intact frogs.

Littlejohn and Oldham (1968) studied the mating call characteristics of members of the Rana pipiens complex

living in the central United States. They identified four different mating call types (Northern, Western, Eastern, and Southern) based on call duration and the temporal characteristics of the pulses within the call (Table 1).

Brown and Brown (1972) recorded the mating calls of members of the Rana pipiens complex in Illinois. Using the classification of mating call types developed by Littlejohn and Oldham (1968), they were able to establish the presence of three of the four call types in Illinois, i.e. Northern, Western, and Eastern call types (Table 2).

The male frogs used in this study were identified morphologically as being the Northern call type of Littlejohn and Oldham (1968). Therefore, the temporal characteristics of the neural correlates of mating calling activity recorded from the isolated brainstem preparations should most closely resemble the temporal characteristics of the Northern call type. This was, indeed, the case. The vocal phase pulse rate of the neural correlates approximated the pulse rate of the Eastern and Northern call types. The vocal phase duration was closest to that of the Northern call type (Table 1). Littlejohn and Oldham recorded the Northern call type at 12° to 16°C but recorded the other three call types at 20° to 25°C. Using the slope of the linear regression formulas developed in this dissertation and reported in Chapter IV, the vocal phase duration and pulse rate values of the neural

Call type	Sample size (individuals)	Pulse rate (No./sec.)	Call duration (seconds)	Temperature
Northern*	4	13.7 (12.9-14.6)	3.75 (3.30-4.73)	12 - 16°C
Western*	7	5.6 (4.6-6.8)	0.66 (0.48-0.89)	20 - 25°C
Eastern*	7	14.8 (14.3-15.3)	0.41 (0.31-0.52)	20 - 25°C
Southern*	7	28.2 (26.0-31.3)	0.64 (9.47-0.83)	20 - 25°C
Neural correlates	42	20.0 (16.2-24.8)	2.35 (1.26-5.79)	20.5°C
Neural correlates**	42	10.4 (6.6-15.2)	3.58 (2.50-7.02)	14.0°C

Table 1. Mating call types of Littlejohn and Oldham in intact frogs compared with neural correlates of mating calling activity in the isolated brainstem.
*Data from Littlejohn and Oldham (1968).
**Corrected to a temperature of 14.0°C.

Call type	Individuals (no.)	Pulse rate (no./sec)	Call duration (sec)	Temperature (°C)
Northern*	5	19.4 (17.0-21.2)	2.90 (2.48-3.39)	17.9 (17.6-18.2)
Western*	2	3.6 (3.6-3.7)	0.69 (0.59-0.78)	18.2 (18.2)
Eastern*	4	9.0 (8.5-9.3)	0.88 (0.74-1.03)	17.7 (17.2-18.2)
Neural correlates**	42	16.3 (12.5-21.1)	2.82 (1.74-6.26)	18.0

Table 2. Mating calls of members of the Rana pipiens complex from Illinois compared with neural correlates of mating calling activity in the isolated brainstems of Rana pipiens pipiens.
*Data from Brown and Brown (1972).
**Corrected to a temperature of 18°C.

correlates recorded at 20.5°C were extrapolated to those expected at 14°C. At this temperature the estimated vocal phase duration became 3.58 seconds and the estimated pulse rate became 10.4 pulses per second. These values were even closer to the values of 3.75 seconds and 13.7 pulses per second given for the Northern call type by Littlejohn and Oldham (1968).

In a similar manner, the call durations and pulse rates reported by Brown and Brown (1972) for the three call types found in Illinois were compared with the data from the 42 isolated brainstem preparations (Table 2). Brown and Brown's recordings were made at approximately 18°C. When the vocal phase duration and the mean pulse rate of the neural correlates of mating calling activity were corrected to 18°C, the correlates most nearly resembled those of the Northern call type described by Brown and Brown.

CHAPTER IV

THE EFFECTS OF TEMPERATURE

A. Introduction

Preliminary temperature experiments were done to determine if the effects of temperature on neural correlates of mating calling activity were similar to the reported effects of temperature on the mating calls of intact frogs. From the preliminary experiments, four hypotheses were proposed:

1) a high positive correlation exists between temperature and pulse rate,
2) a negative correlation exists between temperature and vocal phase duration,
3) no significant correlation exists between temperature and the total number of pulses per call,
4) no significant correlation exists between stimulus-response latency and temperature.

B. Methods

To test these four hypotheses, three experiments using Rana pipiens pipiens purchased from Hazen, Inc. were done in April and May. In each experiment, the ventral

approach was used. The temperature was controlled by the method described in Chapter II.

The neural correlates of mating calling were recorded from the short laryngeal nerve at every 0.5 C^{o} interval from 18.0^{o} to 24.0^{o}C and then from 24.0^{o} to 18.0^{o}C. This temperature range is well within the range over which mating calling occurs in leopard frogs (Blair, 1961). Five to ten minutes would elapse from the beginning of the recording at one temperature interval to the beginning of the recording at the next temperature interval.

The five variables of calling that were quantitated were initial pulse rate, vocal phase pulse rate, vocal phase duration, total pulses per vocal phase, and stimulus-response latency.

Correlation coefficients (r) were used to determine the degree of relationship between temperature and each variable of calling. The Pearson Product-Moment Method was used to calculate this value. The formula used was $r = \frac{\Sigma xy}{\sqrt{\Sigma(x^2)\ \Sigma(y^2)}}$ where x and y = the deviation from $\bar{X}$ (mean of the X scores) and $\bar{Y}$ (mean of the Y scores), respectively. The t test was used to determine the significance of each correlation coefficient. The value of "t" in the t test was determined by $t = \frac{r\sqrt{n - 2}}{\sqrt{1 - r^2}}$ where n = the number of paired xy values.

The numerical value of a correlation coefficient is a measure of the degree of relationship between two variables. The terminology used to describe the degree of this relationship was:

r = 0.00 to 0.20 : negligible

r = 0.20 to 0.40 : low

r = 0.40 to 0.60 : moderate

r = 0.60 to 0.80 : moderately high

r = 0.80 to 0.90 : high

r = 0.90 to 1.00 : very high.

Linear regression lines were used to show the degree of change in one dependent variable (Y_c) occurring in synchrony with a given change in the independent variable (X). Whenever a significant correlation was found between two variables, a regression line was determined, as for example the relationship between temperature and pulse rate. The regression line was expressed by the equation $Y_c = a + bX$. The slope "b" was determined by the expression $b = \frac{r\ (S_Y)}{(S_X)}$. S_Y was the standard deviation of the Y scores. S_X was the standard deviation of the X scores. The Y intercept "a" was determined by the expression $a = \overline{Y} - b\overline{X}$. $\overline{Y}$ was the mean of the Y scores. $\overline{X}$ was the mean of the X scores.

C. Results

1. Initial pulse rate

The correlation between temperature and the initial pulse rate was determined separately for each of the three experiments. The resulting correlation coefficients of $r = 0.990$, $r = 0.985$, and $r = 0.994$ were all significant at the 0.001 level and indicated a very high degree of relationship between temperature and initial pulse rate within each individual brainstem preparation (Fig. 15).

The data from the three experiments were combined yielding a correlation coefficient of 0.848 which was significant at the 0.001 level (Fig. 16). A correlation coefficient of this magnitude indicated a high degree of relationship between the initial pulse rate of neural correlates of mating calling and the temperature of the isolated brainstem preparation.

The linear regression line for the initial pulse rate (Fig. 16) was determined for the combined data using the least squares method. This line was defined by $Y_c = -6.84 + 1.38X$ where Y_c was the best estimate of initial pulse rate at a given temperature represented by X. This data showed that a linear relationship existed between initial pulse rate and temperature such that every Celsius degree increase (or decrease) in temperature resulted in a 1.38 pulses per second increase (or decrease) in pulse rate. Thus, hypothesis 1 was proven true for the initial pulse rate.

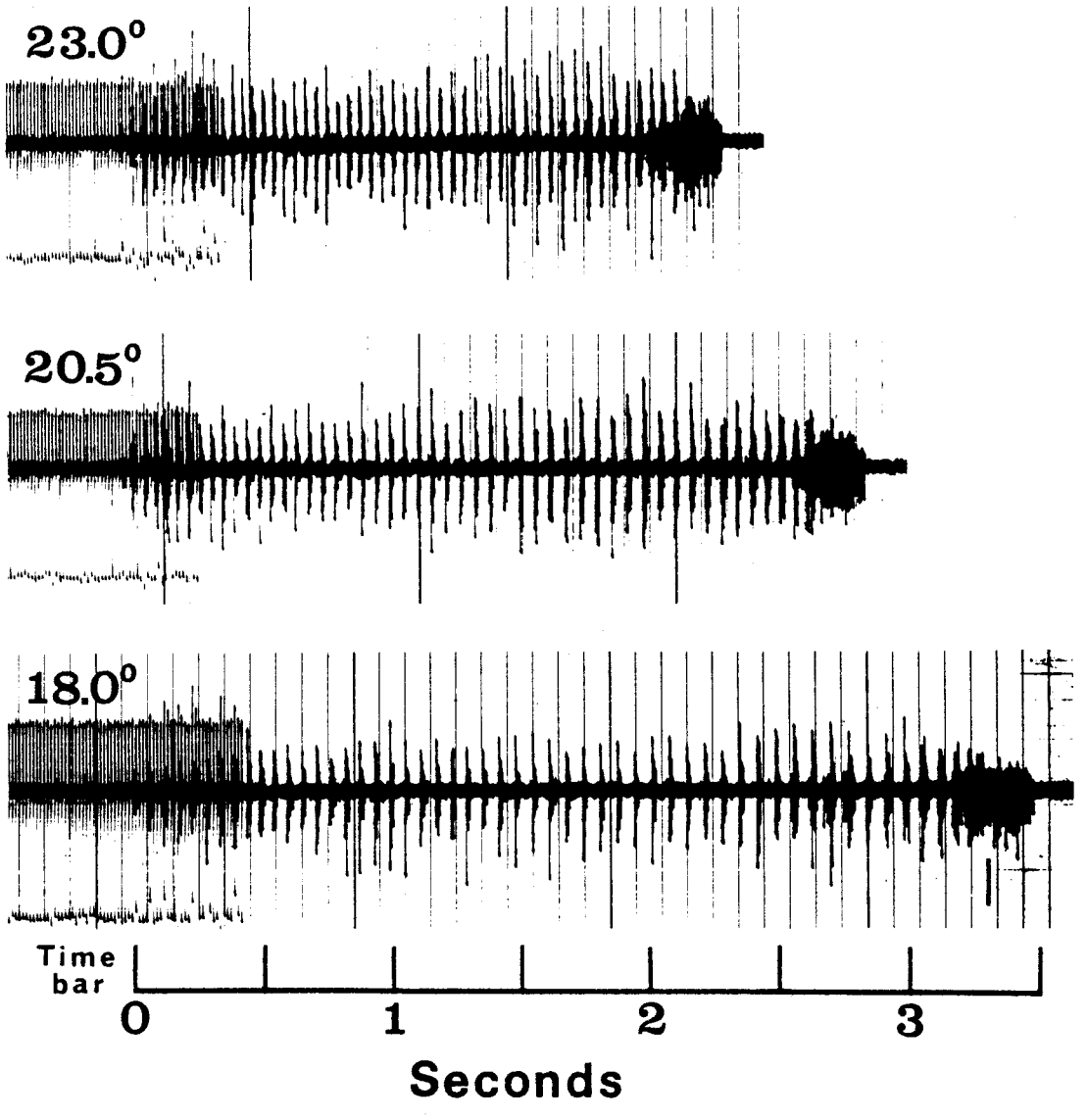

Fig. 15. Effects of temperature on neural correlates of electrically evoked mating calling activity recorded from the same isolated frog brainstem. Temperature of the isolated brainstem at time of recording is shown in upper left of each oscillogram. Horizontal time bar shows passage of time from start of each vocal phase. Oscillograms showed that vocal phase duration increased and pulse rate decreased as temperature dropped from 23.0°C to 18.0°C. Amplitude scale (lower right of bottom oscillogram) = 100 μV.

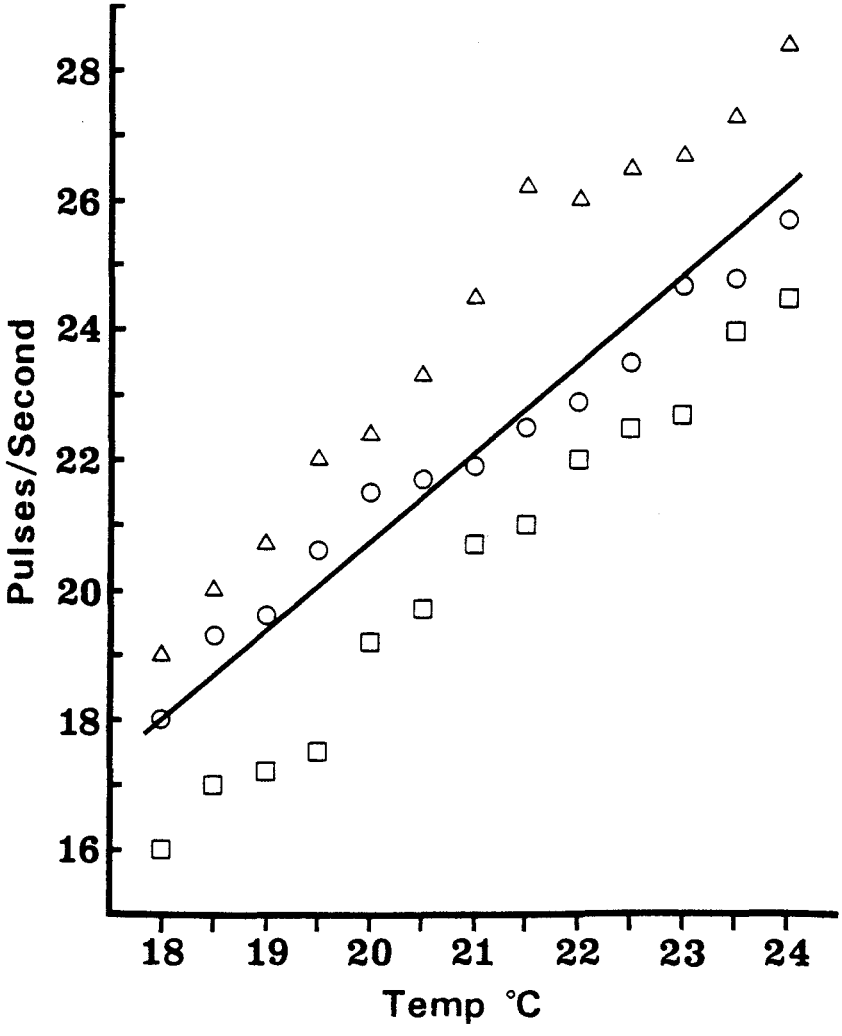

Fig. 16. The effect of temperature on the initial pulse rate of electrically evoked mating calling activity in three isolated frog brainstems. A highly significant ($p < 0.001$) positive correlation ($r = 0.848$) existed between initial pulse rate and temperature. The linear regression line depicting this relationship was $Y_c = -6.84 + 1.38X$.

2. Vocal phase pulse rate

Correlation coefficients calculated for each of the three experiments were $r = 0.985$, $r = 0.990$, and $r = 0.987$. All three were significant at the 0.001 level and indicated a very high degree of relationship between vocal phase pulse rate and temperature within each individual brainstem preparation (Fig. 15).

The correlation coefficient resulting from combining the data (Fig. 17) from all three experiments yielded a positive correlation of 0.897. This correlation was significant at the 0.001 level and indicated a high degree of relationship between vocal phase pulse rate and temperature. The linear regression line for the combined data was $Y_c = -10.36 + 1.48X$.

Again, hypothesis 1 was proven correct.

3. Vocal phase duration

Calculations of vocal phase duration showed a highly significant ($p < 0.001$) negative correlation between vocal phase duration and temperature for two experiments (Fig. 15) and a significant ($p < 0.01$) negative correlation for the third experiment. These correlation coefficients were $r = -0.908$, $r = -0.911$, and $r = -0.736$.

The combined data of the three experiments yielded a highly significant ($p < 0.001$) negative correlation coefficient of -0.626 (Fig. 18). This indicated a moderately

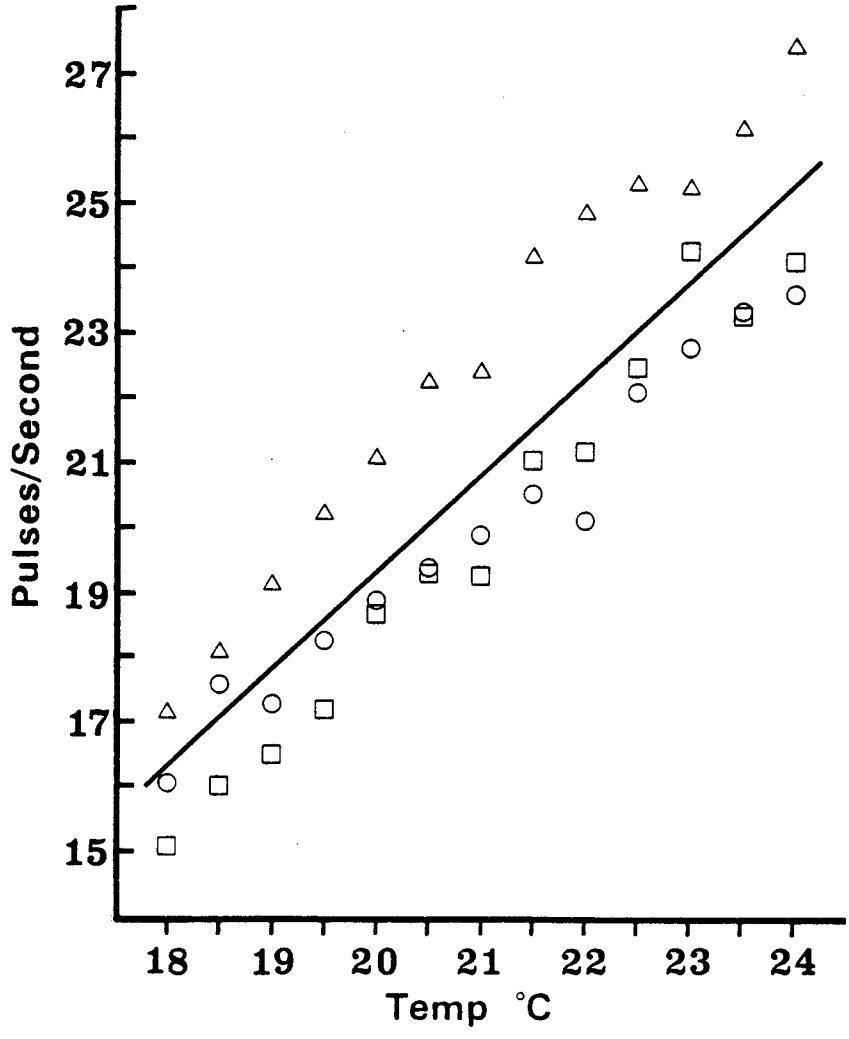

Fig. 17. The effect of temperature on the vocal phase pulse rate of electrically evoked mating calling activity in three isolated frog brainstems. A highly significant ($p < 0.001$) positive correlation ($r = 0.897$) existed between vocal phase pulse rate and temperature. The linear regression line depicting this relationship was $Y_c = -10.36 + 1.48X$.

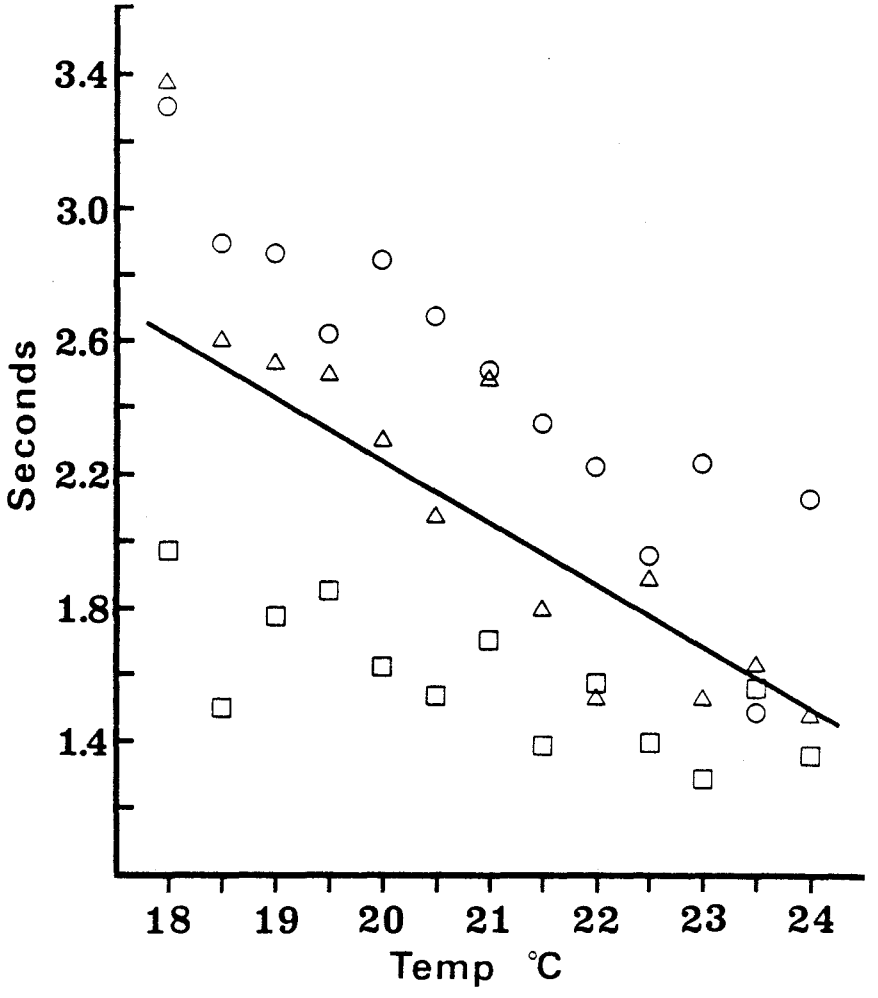

Fig. 18. The effect of temperature on the vocal phase duration of electrically evoked mating calling activity in three isolated frog brainstems. A highly significant ($p < 0.001$) negative correlation ($r = -0.626$) existed between vocal phase duration and temperature. The linear regression line depicting this relationship was $Y_c = 6.00 - 0.19X$.

high degree of relationship. The linear regression line for vocal phase duration was $Y_c = 6.00 - 0.19X$. Thus, every degree increase (or decrease) in temperature resulted in a decrease (or increase) of vocal phase duration of 0.19 seconds.

Hypothesis 2, therefore, was proven to be correct.

4. Total pulses per vocal phase

Calculations of total pulses per vocal phase failed to show a significant correlation coefficient at the $p < 0.01$ level in any of the three individual experiments. The combined data, shown in the scatter diagram in Fig. 19, had a correlation coefficient of -0.183 that was insignificant at the 0.05 level. This low correlation indicated a negligible relationship between temperature and total pulses per vocal phase.

The low correlation provided evidence that hypothesis 3, a null hypothesis, was true.

5. Stimulus-response latency

Two of the three experiments showed a negative correlation between temperature and stimulus-response latency significant at the 0.01 level. The third experiment showed a negative correlation significant at the 0.05 level.

The correlation coefficient of -0.392 for the

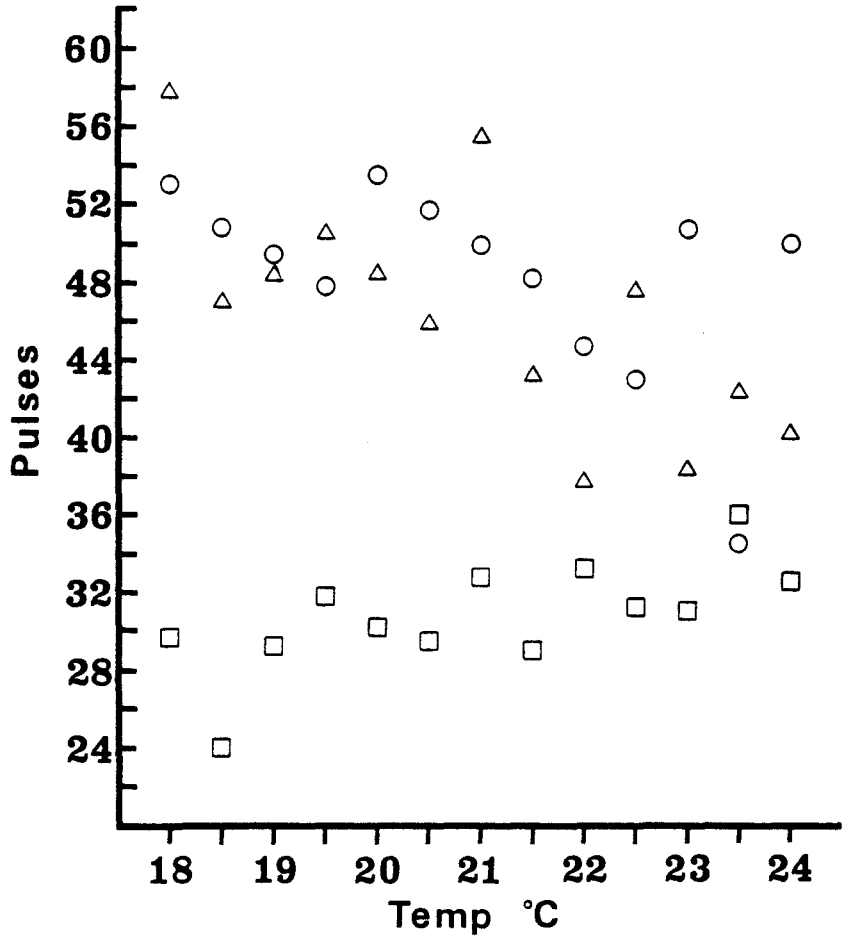

Fig. 19. The effect of temperature on the total pulses per vocal phase of electrically evoked mating calling activity in three isolated frog brainstems. No significant correlation was found.

combined data of all three experiments (Fig. 20) was significant at the 0.05 level and indicated a low degree of relationship. The linear regression line for stimulus-response latency was $Y_c = 6.09 - 0.21X$ which has little value for prediciting latency at a given temperature due to the low degree of relationship.

Hypothesis 4, a null hypothesis, was thus proven wrong at the 0.05 level of significance.

D. Discussion

1. Pulse rate versus temperature

Several investigators have studied the effects of temperature on anuran calling. Littlejohn (1976) reported a highly significant positive relationship between call pulse rate and temperature for Litoria ewingi and L. paraewingi. Brown and Littlejohn (1972) found a highly significant ($p < 0.001$) positive correlation between cloacal temperature and pulse rates of release vibrations and release chirps in the two toads Bufo woodhousei and B. houstonensis. Michaud (1964) reported that an increase in water temperature resulted in an increase in pulse rate of the chorus frogs Pseudacris clarki and P. nigrita. As with the data on intact frogs and toads reported in the literature, the three experiments using isolated frog brainstem preparations showed a highly significant ($p < 0.001$) positive correlation ($r = 0.897$) between temperature

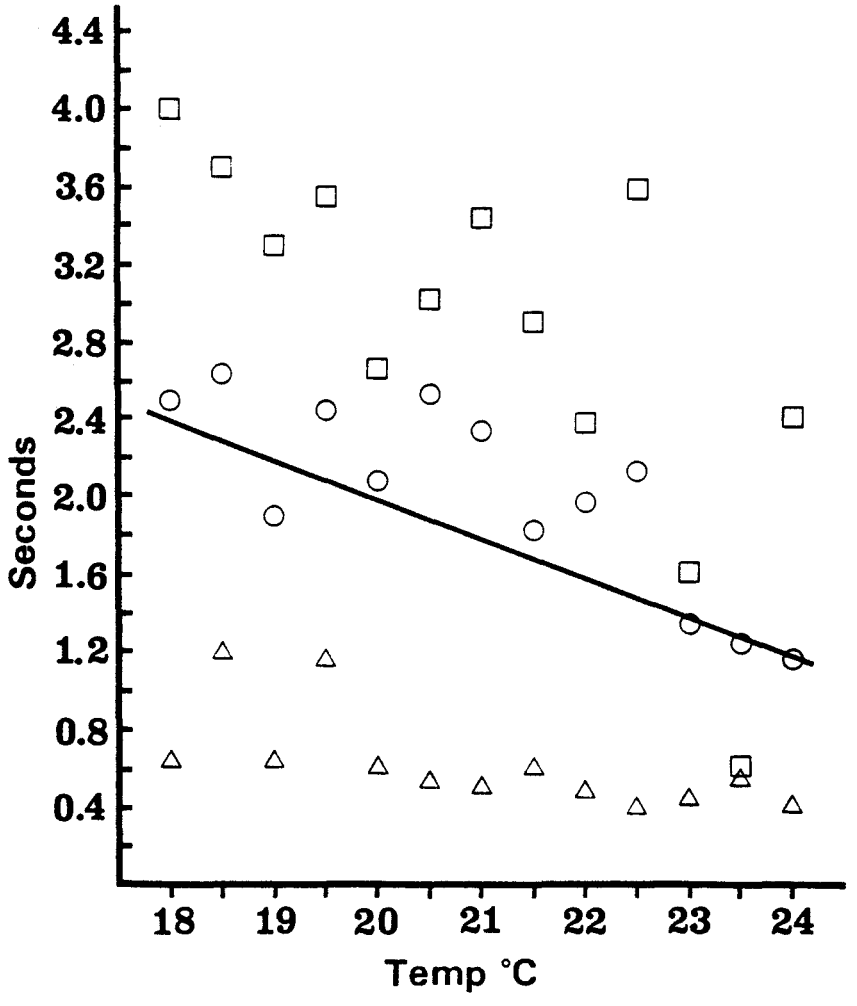

Fig. 20. The effect of temperature on the stimulus-response latency of electrically evoked mating calling activity in three isolated frog brainstems. A significant ($p < 0.05$) negative correlation ($r = -0.392$) existed between stimulus-response latency and temperature. The linear regression line was $Y_c = 6.09 - 0.21X$.

and vocal phase pulse rate of calling.

A number of factors may account for the effects of temperature on pulse rate. For example, temperature may alter the firing rate of an endogenous pacemaker or change the speed of axonal conduction. Temperature may affect a number of physical and chemical processes occurring at synapses. At present, too little is known about the basic neural components responsible for generating the mating calling pattern to explain the effects of temperature on pulse rate.

2. Vocal phase duration versus temperature

Brown and Littlejohn (1972) found a significant ($p < 0.01$) negative correlation between temperature and release vibration duration in Bufo woodhousei and B. houstonensis. Michaud (1964) reported that in the chorus frogs Pseudacris clarki and P. nigrita an increase in water temperature resulted in a decrease in call duration. Schneider (1976) reported that local heating of the head of Bombina bombina caused a decrease in call duration.

The temperature effects on vocal phase duration observed in the isolated frog brainstem preparations showed results similar to those reported in the literature. A highly significant ($p < 0.001$) inverse relationship ($r = -0.63$) was found to exist between temperature and vocal phase duration.

The observed decrease in vocal phase duration with an increase in temperature would be expected if indeed the vocal phase pulse rate increases with an increase in temperature and the total pulses per vocal phase remain constant over the temperature range studied.

3. Total pulses per vocal phase versus temperature

Littlejohn (1976) found no significant relationship between temperature and total number of pulses per call in his field studies of Litoria ewingi and L. paraewingi. Michaud (1964) found that the total number of pulses per call given by Pseudacris clarki and P. nigrita were the same over the entire temperature range studied. Likewise, the isolated frog brainstem preparation showed no significant relationship between temperature and total pulses per vocal phase.

The total pulses per vocal phase may be programmed into the medullary calling circuits. Once the medullary calling circuits have generated the required number of pulses, the vocal phase would end.

4. Stimulus-response latency versus temperature

The temperature effects on stimulus-response latency have not been reported in the literature. In the isolated frog brainstem technique, although there was a significant ($p < 0.05$) inverse relationship between tem-

perature and stimulus-response latency, the correlation coefficient ($r = -0.39$) indicated that this relationship was of a low degree and of little use in prediciting stimulus-response latency based on temperature.

The slightly significant decrease in stimulus-response latency with an increase in temperature may be due to increased axonal conduction velocities. Such temperature effects on conduction velocities have been observed in poikilotherms. For example, as temperature increases, conduction velocities in the median and lateral giant fibers of the earthworm, Lumbricus terrestris, increases (Lagerspetz and Talo, 1967).

5. Temperature effects on auditory system

Hubl, Mohneke, and Schneider (1977) found that the auditory system as well as the calling system of anurans is affected by temperature. They reported an increase in temperature lowered the auditory thresholds in Rana ridibunda ridibunda and Bombina variegata variegata, two Central European anurans. Gerhardt (1978) demonstrated temperature-coupling in gray tree frogs by showing that gravid females, tested at two different temperatures, preferred synthetic mating calls that have temporal properties similar to those produced by male tree frogs at nearly the same temperature as the females. In other words, as the pulse rate of the mating calls of

males increase with temperature, so does the pulse rate preference of the female tree frogs increase.

6. Linear regression lines

Both linear regression lines and Q_{10} values have been used in the literature to represent effects of temperature on a number of factors controlled by poikilotherm systems. Walker (1975a, 1975b) found that because most changes he studied in poikilotherms were linear, these changes were described better by linear regression lines than by Q_{10} values. Unlike linear regression lines, Q_{10} values imply that changes are an exponential function of temperature. Walker observed a linear relationship between wingstroke rate and temperature for numerous species of katydids and crickets. He used linear regression lines to represent the effects of temperature on the electric organ discharge rate in electric fish, the conduction velocity of median and lateral fibers in earthworms, and the stationary discharge rates in crayfish stretch receptors. Gerhart (1978) analyzed the pulse rate of mating calls of 39 male gray tree frogs at body temperatures between 12.8° and 26°C using linear regression lines. Michaud (1964) used linear regression lines to plot call duration of chorus frogs against temperature and pulse rate against temperature.

7. Localization of temperature effects

Schneider (1976) reported the effects of local heating of Bombina bombina, the fire-bellied toad, on the calling pattern. He used a focused spot of light 6 mm in diameter, generated by a modified microscope lamp. He aimed the light on different areas of the toad's body and noted the resulting calls. He found that heating the toad's head always resulted in the most rapid and pronounced changes in calling. Increasing the temperature of the toad's head caused an increase in calling rate (i.e. calls per minute) and a decrease in call duration. Schneider concluded that the major effects of heating on the calling rate and call duration were confined to centers in the central nervous system.

The results of the temperature experiments using the isolated frog brainstem preparation support the contention of Schneider (1976) that temperature alters calling primarily by its effect on the central nervous system since only the central nervous system is used in the isolated frog brainstem preparation.

CHAPTER V

DRUG STUDIES

A. Introduction

To study the feasibility of the use of the isolated brainstem technique for future drug studies of the central generation of mating calling, the flow-through tissue bath described in Chapter II was used because it enables drugs to be introduced readily and removed rapidly from the tissue bath by switching from one leveling bulb solution to another. The drug to be studied was dissolved in measured concentrations in frog saline and placed in one leveling bulb. Another leveling bulb was filled with drug-free frog saline. Two surgical techniques (both described in Chapter II) were used. Initially, the dorsal approach was used with the bipolar stimulating electrode. Later, as the project evolved, the ventral approach with the concentric stimulating electrode was used. No differences in the type or quality of recordings were noted in the two surgical approaches so data from each method were not differentiated but were pooled together. In all studies, the tissue bath was maintained at 20.5°C.

B. Method

1. Experimental design

Electrically evoked mating calling patterns were recorded at fixed intervals (usually two-minute intervals) during a pre-drug control phase, a drug phase, and a post-drug control phase. The resulting neural correlates recorded during the three phases were then quantitated and four variables of calling were compared. The four variables were 1) initial pulse rate, 2) vocal phase duration, 3) total pulses per vocal phase, and 4) stimulus-response latency.

a. Pre-drug control phase

During the pre-drug control phase, oxygenated drug-free frog saline flowed through the tissue bath. Recordings of neural correlates of mating calling were made after the preoptic area of the brainstem was electrically stimulated seven times at two minute intervals. At the end of the seventh recording, the flow of drug-free saline was stopped and the flow of an oxygenated, drug saline solution was started.

b. Drug phase

The oxygenated drug-saline solution was allowed to flow into the tissue bath for ten minutes before

stimulating the brain. Preliminary experiments suggested that maximum drug effects were observed after the drug had been flowing through the tissue bath for ten minutes. Thus, it is believed that the time lapse allows the drug-saline solution to completely replace the drug-free saline in the tissue bath and allows the drug to diffuse into the isolated brainstem preparation.

Ten minutes after the introduction of the drug solution, the brainstem was electrically stimulated seven times at two minute intervals.

If calling activity was evoked, the neural correlates were recorded. If no calling activity was evoked, the preoptic area was still stimulated seven times and the absence of the calling pattern was noted.

At the end of the seventh recording, the flow of drug-saline solution was stopped and the flow of drug-free saline was resumed.

c. Post-drug control phase

The oxygenated drug-free saline was allowed to flow into the tissue bath for ten minutes before stimulating the brain, thereby giving the drug-saline solution time to be flushed from the tissue bath and giving the isolated brainstem preparation time to recover from the effects of the drug.

Ten minutes after the beginning of this phase, the

isolated brainstem was again stimulated electrically seven times at two-minute intervals.

If after the seven stimulations no mating calling activity was evoked, electrical stimulation was continued at two-minute intervals until mating calling activity returned. Then seven recordings of mating calling activity were recorded at two-minute intervals. If, however, calling activity still did not return after 30 minutes of intermittent stimulating, the experiment was terminated. All experiments in which electrically evoked mating calling activity disappeared and failed to return were discarded because the cause for the irreversible stoppage of calling could not be determined. While the preparation could have failed because of a drug effect, it could have failed for other reasons as well.

d. Method of comparing three phases of drug studies

Each time a drug was given using the established protocol, a mean value for initial pulse rate, vocal phase duration, total pulses per vocal phase, and stimulus-response latency was determined for each of the three phases of the study. From these values, the per cent change from pre-drug phase to drug phase and the per cent change from pre-drug phase to post-drug phase were calculated.

No statistically significant correlation coefficient was found between log doses of each drug and the per cent change in any of the four variables studied. The per cent changes in the neural correlates of mating calling activity were presented in tables.

2. ED50 determinations

ED50 determinations were done using the ventral surgical approach described in Chapter II. Two cholinergic antagonists (atropine sulfate and d-tubocurarine chloride) and one anti-cholinesterase (eserine salicylate) were tested. The ED50 determinations gave the median effective dose at which each of these drugs reversibly stopped electrically evoked mating calling activity in 50% of the isolated brainstem preparations.

The Litchfield and Wilcoxon (1949) method of determining median effective dose was used. This method required finding a high dose (a molar concentration that reversibly stops electrically evoked mating calling activity in more than 50% but fewer than 100% of the preparations) and a low dose (a molar concentration that reversibly stops mating calling activity in at least one preparation but fewer than 50% of the preparations). Five isolated brainstem preparations were used at each drug dose. The high and low molar concentrations must be converted into log doses. The per cent of effectiveness,

i.e. the per cent of experiments in which calling activity stopped, of each dose must be converted into a probit value by use of a probit table.

The following formulas were used to determine ED50:

$$\text{Slope (b)} = \frac{Y_1 - Y_2}{X_1 - X_2}$$

$$\text{Log ED50} = X_1 + \frac{5.00 - Y_1}{b}$$

ED50 = antilog of log ED50

where X_1 = high log dose

X_2 = low log dose

Y_1 = probit value for per cent effectiveness of X_1

Y_2 = probit value for per cent effectiveness of X_2

To determine the standard deviation (S) of ED50, the following steps were taken:

$$\text{Log S} = \frac{1}{b\sqrt{\frac{n}{2}}}$$

$n = 5$

$$S = \frac{\text{antilog (log ED50 + log S)} - \text{antilog (log ED50 - log S)}}{2}$$

Median effective dose studies were performed after it was found that drug induced changes in calling activity patterns were generally unpredictable. Several drugs, however, when tested at high concentrations, were found to prevent electrically evoked mating calling activity.

When these drugs were washed out of the bath by drug-free saline, the electrically evoked mating calling patterns returned.

3. Selection of drugs

No precedence existed for the selection of drugs to be applied to calling circuits of the isolated frog brainstem. The drugs selected for this study were drugs known to affect neurotransmitters in the brain.

A number of histochemical studies have reported the presence of noradrenergic, dopaminergic, and serotinergic neurons in the frog brain (Rao and Hartwig, 1974; Vigh-Teichman, Vigh, and Aros, 1969; Parent, 1973, 1975) and in tadpoles (Terlou and Ploemacher, 1973). In the frog Rana temporaria Parent (1973) found catecholamine (CA)-type cell bodies concentrated near the midline at the mesodiencephalic junction in the preoptic recess organ, the paraventricular organ, and the lateral recess region. CA cells were also scattered in the reticular formation of the lower brainstem. Serotonin-type neurons were found concentrated within the midbrain tegmentum and within the raphe region in a cell column extending from the caudal half of the midbrain tegmentum to the level of the lower medulla. Based on these histochemical studies, a few experiments were done with a small number of drugs known to affect the noradrenergic, dopaminergic, and serotin-

ergic systems. Preliminary studies with these drugs were inconclusive.

Acetylcholine (ACh) has been implicated as a possible neurotransmitter in the frog brain by several investigators using different research methods. Hebb and Ratkovic (1964), in measuring the concentrations of choline acetylase in representatives of most major groups of vertebrate, found this enzyme in the brain of Rana temporaria. The level was highest in the hindbrain. Choline acetylase (choline acetyltransferase) catalyzes the synthesis of ACh from acetyl CoA and choline. Therefore, the presence of this enzyme is suggestive that ACh is present and functional in the frog brain.

ACh was bioassayed in three regions of the brain of Rana esculenta using the dorsal muscle of the leech (Nistri, DeBellis, and Cammelli, 1975; Cammelli, DeBellis, and Nistri, 1974). The highest concentrations of ACh were found in the diencephalon and mesencephalon. The second highest concentrations were measured in the rhombencephalon. The lowest concentrations were found in the telencephalon, but even the concentrations here were relatively high compared to levels found in the mammalian brain.

Acetylcholinesterase (AChE) activity was measured in three regions of the brain of R. esculenta spectrophotometrically using acetylthiocholine as a substrate

(Nistri, DeBellis, and Cammelli, 1975; Cammelli, DeBellis, and Nistri, 1974). The midbrain and rhombencephalon had similar levels of activity. The telencephalon had the lowest AChE activity. Another distribution study of AChE used a direct chemical assay (manometric technique) and histochemical technique to measure AChE in the brain of R. p. pipiens (Shen, Greenfield, and Boell, 1955). The AChE was found to be highest in the mesencephalon and was moderately high in the diencephalon and medulla. A high concentration was found in the motor neurons of cranial nerves V, VII, and X.

These reports of measurable levels of choline acetylase, ACh, and AChE led to the selection of cholinergic drugs for testing. Initial studies seemed promising; therefore, emphasis was placed on studies of drugs affecting the cholinergic system.

Although cholinergic pathways have not been worked out in any detail in the frog's brain, some information about these pathways in mammals exists. Histochemical studies have identified cholinesterase-containing pathways in the rat brain. A dorsal tegmental pathway arises from the cuneiform nucleus of the mesencephalic reticular formation and ascends, in a diffuse manner, to the tectum, pretectal area, geniculate bodies, and thalamus. A ventral tegmental pathway emanates from the substantia nigra and ventral tegmental area and projects to the globus

pallidus, caudate nucleus, putamen, and also to the limbic cortex and medial septum. The medial septum sends cholinergic afferents to the hippocampus. Another cholinergic system in the rat has bi-directional pathways connecting the hippocampus with the septum, thalamus, preoptic area, and mammillary bodies. There are also connections to midbrain structures (as reviewed by Iversen and Iversen, 1975; Myers, 1974).

The only reports in the literature of the use of drugs to affect the central generation of mating calling is the use of hormones. Schmidt (1966b) injected macerated anterior pituitaries from R. p. pipiens into other R. p. pipiens to bring them into a state of readiness to call. In such a state, males answered a tape recording of a conspecific mating call by giving a mating call. Schmidt also brought certain tree frogs into calling condition by injections of human chorionic gonadotropin as well as by injections of anterior pituitary preparations. While these studies involved the giving of drugs to induce a readiness to call, they did not deal with the use of drugs to alter the generation of the calling pattern.

C. Results

1. Sham drug

Sham drug experiments were run on four isolated

brainstem preparations using the same format as was used for the other drug experiments. Both leveling bulbs were filled with oxygenated drug-free frog saline.

The purpose of the sham drug experiments was to investigate in the absence of any drugs, the per cent change that occurs in the neural correlates of mating calling between phases of the experiment. The results of the sham drug experiments are listed in Tables 3-6. Tables 7 and 8 give the largest single change, the mean absolute change, and the mean change between the pre-drug to drug phase and the pre-drug to post-drug phase for all four variables.

The initial pulse rate changed the least during the experiments. Between both the pre-drug to drug phase and the pre-drug to post-drug phase the mean absolute change in pulse rate was less than 3% (Tables 7 and 8). When the direction of change was considered, the decreases in pulse rate cancelled out most of the increases in pulse rate. The result was a change of only 0.8% from the pre-drug to drug phase and only 1.0% from the pre-drug to post-drug phase. The largest single change in pulse rate occurred in Experiment 1 with a change of 7% from the pre-drug phase to the post-drug phase.

In sharp contrast to the initial pulse rate, the other three variables resulted in much larger changes during the sham drug experiments as summarized in Tables

Table 3. Per cent change in initial pulse rate during sham drug experiments

Exp no.	Pre-drug phase to drug phase	Pre-drug phase to post-drug phase
1	6	7
2	-1	1
3	-3	-4
4	1	0

Table 4. Per cent change in vocal phase duration during sham drug experiments

Exp no.	Pre-drug phase to drug phase	Pre-drug phase to post-drug phase
1	-32	-25
2	12	-1
3	15	-7
4	-22	-33

Table 5. Per cent change in total pulses per vocal phase during sham drug experiments

Exp no.	Pre-drug phase to drug phase	Pre-drug phase to post-drug phase
1	-28	-19
2	13	0
3	9	-13
4	-19	-32

Table 6. Per cent change in stimulus-response latency during sham drug experiments

Exp no.	Pre-drug phase to drug phase	Pre-drug phase to post-drug phase
1	10	45
2	19	31
3	16	-20
4	-5	26

Table 7. Summary of per cent change in four variables of neural correlates during pre-drug to drug phase in four sham drug experiments

Variable	Largest single change	Mean absolute* change	Mean change
Initial pulse rate	6	2.7	0.8
Vocal phase duration	-32	20.2	-6.8
Total pulses per vocal phase	-28	17.2	-6.2
Stimulus-response latency	19	12.5	10.0

*Absolute = numerical value irrespective of sign.

Table 8. Summary of per cent change in four variables of neural correlates during pre-drug to post-drug phase in four sham drug experiments

Variable	Largest single change	Mean absolute* change	Mean change
Initial pulse rate	7	2.8	1.0
Vocal phase duration	-33	16.5	-16.5
Total pulses per vocal phase	-32	16.0	-16.0
Stimulus-response latency	45	30.5	20.5

*Absolute = numerical value irrespective of sign.

7 and 8. The smallest mean absolute change in the remaining three variables was 12.5%. This was more than four times larger than the mean absolute change in pulse rate. The smallest mean change that occurred when the direction of change was considered was -6.2%, more than six times larger than the largest mean change in initial pulse rate.

The sham drug data aided in interpretation of drug data. Initial pulse rate varied little during the sham drug experiments; therefore small drug-induced changes in this variable should be apparent. On the other hand, because vocal phase duration, total pulses per vocal phase, and stimulus-response latency changed a great deal during the sham drug experiments, a change had to be relatively large to be interpreted as drug-induced; therefore small drug-induced changes would not be distinguishable from normal variabilities in the preparation.

2. Eserine salicylate

a. Mechanism of action

Eserine salicylate is an anticholinesterase agent. It competes with ACh for binding to AChE. Eserine binds to AChE forming a complex that is hydrolyzed much more slowly than the complex that is formed between ACh and AChE. It effectively reduces the amount of AChE available to terminate the transmitter action of ACh. ACh thus

accumulates at cholinergic sites. The effects of eserine and other anticholinesterases on the central nervous system are reviewed by Karczmar (1970).

b. Effect on mating calling pattern

Eserine salicylate (Sigma) was given to 17 isolated brainstem preparations in doses ranging from 1×10^{-3}M to 1×10^{-6}M. The results are shown in Tables 9-12.

Neural correlates of mating calling activity were electrically evoked during the drug phase in 11 out of 17 isolated brainstem preparations. In all 11 preparations eserine consistently decreased the initial pulse rate. The degree of reduction in pulse rate was not, however, related to the concentration of eserine in the tissue bath. After eserine was rinsed from the bath, the pulse rate increased in nine of the 11 preparations.

The effect of eserine on the vocal phase duration was inconsistent. Vocal phase duration was increased in six preparations and decreased in five preparations. The effect of eserine on total pulses per vocal phase was also inconsistent. The total pulses per call increased in four preparations, stayed the same in one, and decreased in six.

The effect of eserine on stimulus-response latency was consistent in the direction of change, i.e. stimulus-

Table 9. Per cent change in initial pulse rate during eserine salicylate experiments

Drug concn	Exp no.	Pre-drug phase to drug phase	Pre-drug phase to post-drug phase
1×10^{-3}M	1	-19	-16
	2	Stop*	0
	3	Stop	-17
	4	Stop	-5
	5	Stop	-16
5×10^{-4}M	6	-4	-7
	7	-8	3
	8	-8	-11
	9	Stop	-14
	10	Stop	-8
1×10^{-4}M	11	-11	4
	12	-7	-3
5×10^{-5}M	13	-17	-1
1×10^{-5}M	14	-11	-7
	15	-6	1
5×10^{-6}M	16	-1	1
1×10^{-6}M	17	-6	-2

*Stop indicates that no mating calling pattern could be elicited.

Table 10. Per cent change in vocal phase duration during eserine salicylate experiments

Drug concn	Exp no.	Pre-drug phase to drug phase	Pre-drug phase to post-drug phase
1×10^{-3}M	1	37	23
	2	Stop*	38
	3	Stop	25
	4	Stop	27
	5	Stop	8
5×10^{-4}M	6	-8	-43
	7	11	28
	8	33	48
	9	Stop	-26
	10	Stop	108
1×10^{-4}M	11	2	65
	12	46	21
5×10^{-5}M	13	-42	-33
1×10^{-5}M	14	18	21
	15	-9	-19
5×10^{-6}M	16	-10	-4
1×10^{-6}M	17	-7	12

*Stop indicates that no mating calling pattern could be elicited.

Table 11. Per cent change in total pulses per vocal phase during eserine salicylate experiments

Drug concn	Exp no.	Pre-drug phase to drug phase	Pre-drug phase to post-drug phase
1×10^{-3}M	1	17	5
	2	Stop*	34
	3	Stop	1
	4	Stop	24
	5	Stop	0
5×10^{-4}M	6	-7	-42
	7	0	28
	8	24	33
	9	Stop	-30
	10	Stop	94
1×10^{-4}M	11	-13	71
	12	33	12
5×10^{-5}M	13	-45	-26
1×10^{-5}M	14	6	7
	15	-10	-15
5×10^{-6}M	16	-11	-3
1×10^{-6}M	17	-11	8

*Stop indicates that no mating calling pattern could be elicited.

Table 12. Per cent change in stimulus-response latency during eserine salicylate experiments

Drug Concn	Exp no.	Pre-drug phase to drug phase	Pre-drug phase to post-drug phase
1×10^{-3}M	1	78	106
	2	Stop*	31
	3	Stop	158
	4	Stop	41
	5	Stop	-44
5×10^{-4}M	6	5	70
	7	108	24
	8	95	109
	9	Stop	-24
	10	Stop	139
1×10^{-4}M	11	70	30
	12	18	14
5×10^{-5}M	13	11	-25
1×10^{-5}M	14	15	-23
	15	72	-2
5×10^{-6}M	16	4	22
1×10^{-6}M	17	95	44

*Stop indicates that no mating calling pattern could be elicited.

response latency increased in all 11 preparations, but the increased latency was not dose related.

c. ED50 determination

At a concentration of 1×10^{-3}M, eserine salicylate reversibly stopped the generation of the mating calling pattern in four out of five isolated brainstem preparations. The lower concentrations of 5×10^{-4}M eserine salicylate reversibly stopped mating calling activity in two of five preparations. The ED50 of eserine salicylate was 5.90×10^{-4}M with a standard deviation of 2.30×10^{-4}M.

3. Atropine sulfate

a. Mechanism of action

Atropine sulfate is an antimuscarinic drug. It competes with ACh for binding to the muscarinic receptor. It inhibits the action of ACh on structures innervated by postganglionic cholinergic nerves and on smooth muscles that respond to ACh. In the brain of vertebrates a majority of the excitatory and all of the depressant effects of ACh appear to be due to the action of ACh on muscarinic receptors. Atropine blocks these muscarinic effects (Krnjević, 1974).

b. Effects on the mating calling pattern

Atropine sulfate (Sigma) was given to 14 isolated brainstem preparations. The concentrations ranged from 1×10^{-3}M to 1×10^{-4}M. The results of atropine administration are given in Tables 13-16.

In eight of the 14 experiments neural correlates of mating calling activity were still evoked during the drug phase by electrical stimulation of the preoptic area. Vocal phase duration and total pulses per vocal phase decreased during the drug phase in seven of eight experiments. The initial pulse rate decreased in five of the eight experiments and latency decreased in four of the eight. There was not, however, a graded dose-response relationship. There was also not a consistent trend in the pre-drug to post-drug phase.

c. ED50 determination

The high dose of atropine, 5×10^{-4}M, reversibly stopped mating calling activity in four of the five isolated brainstem preparations. The low dose, 3.16×10^{-4}M, reversibly stopped calling in one out of five preparations. The ED50 of atropine sulfate was 3.98×10^{-4}M with a standard deviation of 1.9×10^{-5}M.

Table 13. Per cent change in initial pulse rate during atropine sulfate experiments

Drug concn	Exp no.	Pre-drug phase to drug phase	Pre-drug phase to post-drug phase
1×10^{-3}M	1	Stop*	-17
5×10^{-4}M	2	-32	-20
	3	Stop	-16
	4	Stop	-2
	5	Stop	-3
	6	Stop	-15
3.16×10^{-4}M	7	5	-2
	8	-2	-4
	9	-1	-9
	10	1	-3
	11	Stop	8
1×10^{-4}M	12	5	2
	13	-4	-2
	14	-2	-8

*Stop indicates that no mating calling pattern could be elicited.

Table 14. Per cent change in vocal phase duration during atropine sulfate experiments

Drug Concn	Exp no.	Pre-drug phase to drug phase	Pre-drug phase to post-drug phase
1×10^{-3}M	1	Stop*	-55
5×10^{-4}M	2	-23	-16
	3	Stop	-58
	4	Stop	-4
	5	Stop	-29
	6	Stop	-78
3.16×10^{-4}M	7	-61	-36
	8	1	27
	9	-7	2
	10	-44	-51
	11	Stop	79
1×10^{-4}M	12	-13	-22
	13	-11	3
	14	-23	-28

*Stop indicates that no mating calling pattern could be elicited.

Table 15. Per cent change in total pulses per vocal phase during atropine sulfate experiments

Drug concn	Exp no.	Pre-drug phase to drug phase	Pre-drug phase to post-drug phase
1×10^{-3}M	1	Stop	-60
5×10^{-4}M	2	-46	-31
	3	Stop*	-63
	4	Stop	-7
	5	Stop	-22
	6	Stop	-79
3.16×10^{-4}M	7	-57	-37
	8	1	21
	9	-8	-6
	10	-41	-50
	11	Stop	83
1×10^{-4}M	12	-8	-17
	13	-12	0
	14	-22	-30

*Stop indicates that no mating calling pattern could be elicited.

Table 16. Per cent change in stimulus-response latency during atropine sulfate experiments

Drug concn	Exp no.	Pre-drug phase to drug phase	Pre-drug phase to post-drug phase
1×10^{-3}M	1	Stop*	125
5×10^{-4}M	2	27	0
	3	Stop	-36
	4	Stop	-7
	5	Stop	61
	6	Stop	-10
3.16×10^{-4}M	7	8	20
	8	-11	-1
	9	-62	-38
	10	-59	4
	11	Stop	32
1×10^{-4}M	12	9	15
	13	4	16
	14	-18	-8

*Stop indicates that no mating calling pattern could be elicited.

4. d-Tubocurarine chloride

a. Mechanism of action

d-Tubocurarine chloride is a competitive nicotinic blocking agent. It competes with ACh for the cholinergic (nicotinic) receptor site on the postjunctional membranes of striated muscles. Once it combines with the cholinoceptive site, it prevents the transmitter action of ACh. Tubocurarine also blocks the nicotinic receptors of the autonomic ganglia. Tubocurarine, possessing two quaternary nitrogens, is generally blocked from entry into the central nervous system by the blood-brain barrier. Once in the central nervous system, it blocks central nicotinic receptors; however Krnjević (1974) feels tubocurarine is not a useful ACh antagonist in the central nervous system because it strongly excites many neurons.

b. Effects on mating calling pattern

d-Tubocurarine chloride (Sigma) was given to 15 isolated brainstem preparations in concentrations ranging from 3.16×10^{-5}M to 1×10^{-6}M. The results are given in Tables 17-20.

During the drug phase, electrically evoked mating calling activity remained in ten of the 15 preparations. All four of the variables studied showed inconclusive changes following the introduction of d-tubocurarine

Table 17. Per cent change in initial pulse rate during d-tubocurarine chloride experiments

Drug concn	Exp no.	Pre-drug phase to drug phase	Pre-drug phase to post-drug phase
3.16×10^{-5}M	1	-9	-5
	2	-11	-16
	3	Stop*	-10
	4	Stop	7
	5	Stop	-13
1×10^{-5}M	6	-14	0
	7	2	-2
	8	7	-7
	9	Stop	-2
	10	Stop	-8
3.16×10^{-6}M	11	-3	6
	12	3	4
1×10^{-6}M	13	2	-5
	14	-2	3
	15	10	12

*Stop indicates that no mating calling pattern could be elicited.

Table 18. Per cent change in vocal phase duration during d-tubocurarine chloride experiments

Drug concn	Exp no.	Pre-drug phase to drug phase	Pre-drug phase to post-drug phase
3.16×10^{-5}M	1	13	-4
	2	-29	-37
	3	Stop*	-29
	4	Stop	-15
	5	Stop	0
1×10^{-5}M	6	-36	38
	7	76	-19
	8	33	-24
	9	Stop	-15
	10	Stop	-18
3.16×10^{-6}M	11	-42	23
	12	15	1
1×10^{-6}M	13	-7	4
	14	5	-27
	15	28	-16

*Stop indicates that no mating calling pattern could be elicited.

Table 1?. Per cent change in total pulses per vocal phase during d-tubocurarine chloride experiments

Drug concn	Exp no.	Pre-drug phase to drug phase	Pre-drug phase to post-drug phase
3.16×10^{-5}M	1	3	-6
	2	-35	-45
	3	Stop*	-35
	4	Stop	-10
	5	Stop	-7
1×10^{-5}M	6	-43	34
	7	84	-18
	8	54	-26
	9	Stop	-16
	10	Stop	-21
3.16×10^{-6}M	11	-38	25
	12	15	4
1×10^{-6}M	13	-7	2
	14	5	-23
	15	35	-21

*Stop indicates that no mating calling pattern could be elicited.

Table 20. Per cent change in stimulus-response latency during d-tubocurarine chloride experiments

Drug concn	Exp no.	Pre-drug phase to drug phase	Pre-drug phase to post-drug phase
3.16×10^{-5}M	1	218	103
	2	-70	-48
	3	Stop*	51
	4	Stop	24
	5	Stop	120
1×10^{-5}M	6	70	-17
	7	-57	-29
	8	52	15
	9	Stop	43
	10	Stop	-20
3.16×10^{-6}M	11	14	73
	12	33	4
1×10^{-6}M	13	124	51
	14	17	6
	15	11	-1

*Stop indicates that no mating calling pattern could be elicited.

chloride. Even the direction of change was inconsistent.

c. ED50 determination

Mating calling activity stopped completely during the drug phase in three of the five preparations at a drug concentration of 3.16×10^{-5}M and in two of the five preparations at a concentration of 1×10^{-5}M. The calculated ED50 value was 1.80×10^{-5}M. The standard deviation was 3.54×10^{-5}M.

5. Gallamine triethiodide

Gallamine triethiodide is a nicotinic blocker with the same mechanism of action as d-tubocurarine chloride.

Gallamine (Flaxedil^{R}, Davis and Geck, American Cyanamid Company) was given to 14 isolated brainstem preparations in concentrations ranging from 5×10^{-5}M to 5×10^{-9}M (Tables 21-24).

When gallamine was given at a concentration of 5×10^{-5}M to two preparations, mating calling activity reversibly stopped. Mating calling activity was reversibly stopped in one of two preparations given gallamine at a concentration of 1×10^{-5}M.

The effects of gallamine on the four variables of the mating calling pattern were inconsistent in the direction of change and not dose related.

Table 21. Per cent change in initial pulse rate during gallamine triethiodide experiments

Drug concn	Exp no.	Pre-drug phase to drug phase	Pre-drug phase to post-drug phase
5×10^{-5}M	1	Stop*	-9
	2	Stop	-10
1×10^{-5}M	3	Stop	-7
	4	-1	-3
1×10^{-6}M	5	5	0
	6	5	6
	7	-4	-3
	8	2	-1
5×10^{-6}M	9	-1	-3
5×10^{-7}M	10	1	-1
1×10^{-7}M	11	-3	-2
	12	3	3
1×10^{-8}M	13	3	3
5×10^{-9}M	14	4	6

*Stop indicates that no mating calling pattern could be elicited.

Table 22. Per cent change in vocal phase duration during gallamine triethiodide experiments

Drug concn	Exp no.	Pre-drug phase to drug phase	Pre-drug phase to post-drug phase
5×10^{-5}M	1	Stop*	-32
	2	Stop	17
1×10^{-5}M	3	Stop	-8
	4	15	0
1×10^{-6}M	5	-10	-24
	6	-25	-25
	7	-2	-6
	8	-5	6
5×10^{-6}M	9	-24	9
5×10^{-7}M	10	-15	4
1×10^{-7}M	11	-56	-37
	12	29	5
1×10^{-8}M	13	-17	-4
5×10^{-9}M	14	-8	-31

*Stop indicates that no mating calling pattern could be elicited.

Table 23. Per cent change in total pulses per vocal phase during gallamine triethiodide experiments

Drug concn	Exp no.	Pre-drug phase to drug phase	Pre-drug phase to post-drug phase
5×10^{-5}M	1	Stop*	-30
	2	Stop	6
1×10^{-5}M	3	Stop	-53
	4	15	-5
1×10^{-6}M	5	-7	-23
	6	-20	-19
	7	-12	-9
	8	-5	2
5×10^{-6}M	9	-21	8
5×10^{-7}M	10	-12	5
1×10^{-7}M	11	-52	-36
	12	27	10
1×10^{-8}M	13	-13	-1
5×10^{-9}M	14	-6	-30

*Stop indicates that no mating calling pattern could be elicited.

Table 24. Per cent change in stimulus-response latency during gallamine triethiodide experiments

Drug concn	Exp no.	Pre-drug phase to drug phase	Pre-drug phase to post-drug phase
5×10^{-5}M	1	Stop*	60
	2	Stop	221
1×10^{-5}M	3	Stop	-30
	4	27	-3
1×10^{-6}M	5	-8	21
	6	-18	-27
	7	-12	12
	8	-1	7
5×10^{-6}M	9	-10	-29
5×10^{-7}M	10	-11	-28
1×10^{-7}M	11	20	-1
	12	16	22
1×10^{-8}M	13	67	30
5×10^{-9}M	14	24	-4

*Stop indicates that no mating calling pattern could be elicited.

6. Mecamylamine hydrochloride

Mecamylamine hydrochloride is a ganglionic (nicotinic) blocker. It can also block other nicotinic receptors when given in higher concentrations. It is a secondary amine and therefore can pass the blood-brain barrier naturally, unlike tubocurarine, another nicotinic blocker used in this project.

Mecamylamine HCl (Sigma) was given to seven isolated brainstem preparations (Tables 25-28). At the highest concentration given (1×10^{-4}M), stimulus-response latency increased. The effects of mecamylamine on the other three variables were inconclusive.

7. Nicotine sulfate

Nicotine sulfate has a dual action on the cholinergic receptors of autonomic ganglia. First, it stimulates the receptors but then at high doses it causes protracted depolarization of the receptors, effectively blocking the transmitter action of ACh. Nicotine also acts on nicotinic receptors in the central nervous system. Its effect on the Renshaw cells of the spinal cord is well-documented (Eccles, 1964).

Nicotine sulfate (Sigma) was given to nine isolated brainstem preparations (Tables 29-32). Nicotine sulfate reversibly stopped mating calling activity in a

Table 25. Per cent change in initial pulse rate during mecamylamine hydrochloride experiments

Drug concn	Exp no.	Pre-drug phase to drug phase	Pre-drug phase to post-drug phase
1×10^{-4}M	1	-27	-23
	2	-3	-4
	3	2	3
5×10^{-5}M	4	4	-5
1×10^{-5}M	5	-1	0
	6	15	15

Table 26. Per cent change in vocal phase duration during mecamylamine hydrochloride experiments

Drug concn	Exp no.	Pre-drug phase to drug phase	Pre-drug phase to post-drug phase
1×10^{-4}M	1	90	129
	2	0	13
	3	0	28
5×10^{-5}M	4	-5	-9
1×10^{-5}M	5	10	0
	6	11	16

Table 27. Per cent change in total pulses per vocal phase during mecamylamine hydrochloride experiments

Drug concn	Exp no.	Pre-drug phase to drug phase	Pre-drug phase to post-drug phase
1×10^{-4}M	1	56	97
	2	0	10
	3	1	31
5×10^{-5}M	4	-2	-8
1×10^{-5}M	5	9	-2
	6	15	19

Table 28. Per cent change in stimulus-response latency during mecamylamine hydrochloride experiments

Drug concn	Exp no.	Pre-drug phase to drug phase	Pre-drug phase to post-drug phase
1×10^{-4}M	1	65	31
	2	46	169
	3	156	212
5×10^{-5}M	4	8	-1
1×10^{-5}M	5	16	3
	6	-6	-19

Table 29. Per cent change in initial pulse rate during nicotine sulfate experiments

Drug concn	Exp no.	Pre-drug phase to drug phase	Pre-drug phase to post-drug phase
1×10^{-3}M	1	-1	2
5×10^{-4}M	2	Stop*	-8
	3	Stop	-6
	4	-3	-1
	5	1	1
2×10^{-4}M	6	Stop	-4
2×10^{-5}M	7	-2	-1
1×10^{-5}M	8	1	12
1×10^{-7}M	9	4	3

*Stop indicates that no mating calling pattern could be elicited.

Table 30. Per cent change in vocal phase duration during nicotine sulfate experiments

Drug concn	Exp no.	Pre-drug phase to drug phase	Pre-drug phase to post-drug phase
1×10^{-3}M	1	-16	-30
5×10^{-4}M	2	Stop*	0
	3	Stop	37
	4	-53	-38
	5	33	42
2×10^{-4}M	6	Stop	-37
2×10^{-5}M	7	-13	-18
1×10^{-5}M	8	42	0
1×10^{-7}M	9	9	16

*Stop indicates that no mating calling pattern could be elicited.

Table 31. Per cent change in total pulses per vocal phase during nicotine sulfate experiments

Drug concn	Exp no.	Pre-drug phase to drug phase	Pre-drug phase to post-drug phase
1×10^{-3}M	1	-12	-25
5×10^{-4}M	2	Stop*	-4
	3	Stop	29
	4	-50	-35
	5	30	41
2×10^{-4}M	6	Stop	-38
2×10^{-5}M	7	-15	-19
1×10^{-5}M	8	36	9
1×10^{-7}M	9	9	17

*Stop indicates that no mating calling pattern could be elicited.

Table 32. Per cent change in stimulus-response latency during nicotine sulfate experiments

Drug concn	Exp no.	Pre-drug phase to drug phase	Pre-drug phase to post-drug phase
1×10^{-3}M	1	0	13
5×10^{-4}M	2	Stop*	23
	3	Stop*	22
	4	23	46
	5	77	-8
2×10^{-4}M	6	Stop	51
2×10^{-5}M	7	21	8
1×10^{-5}M	8	-17	-36
1×10^{-7}M	9	-25	-30

*Stop indicates that no mating calling pattern could be elicited.

single preparation at 2 x 10^{-4}M and in two out of four preparations at 5 x 10^{-4}M. Despite these effects, it failed to prevent mating calling activity during the drug phase in the one preparation to which it was given at the highest concentration of 1 x 10^{-3}M. The effects of nicotine sulfate on the four variables studied were inconsistent in the direction of change and inconclusive.

8. Carbamylcholine chloride

Carbamylcholine chloride, i.e. carbachol, is a potent choline ester that stimulates both muscarinic and nicotinic receptors. It is resistent to hydrolysis by either AChE or nonspecific cholinesterases.

Carbamylcholine chloride (Sigma) was administered to 11 isolated brainstem preparations in concentrations ranging from 1 x 10^{-3}M to 1 x 10^{-7}M. The results appear in Tables 33-36. At the highest dose given, 1 x 10^{-3}M, it reversibly stopped mating calling activity. Mating calling activity was still evoked by electrical stimulation of the preoptic area during exposure to lower concentrations of carbamylcholine chloride. Nevertheless, the results were inconsistent in the direction of change and inconclusive.

9. Norepinephrine bitartrate

Norepinephrine is the neurotransmitter released at

Table 33. Per cent change in initial pulse rate during carbamylcholine chloride experiments

Drug concn	Exp no.	Pre-drug phase to drug phase	Pre-drug phase to post-drug phase
1×10^{-3}M	1	Stop*	9
1×10^{-4}M	2	1	1
	3	-8	-6
	4	-7	-8
5×10^{-4}M	5	2	9
5×10^{-5}M	6	-8	-6
	7	0	1
	8	9	19
1×10^{-5}M	9	1	3
1×10^{-6}M	10	1	2
1×10^{-7}M	11	2	5

*Stop indicates that no mating calling pattern could be elicited.

Table 34. Per cent change in vocal phase duration during carbamylcholine chloride experiments

Drug concn	Exp no.	Pre-drug phase to drug phase	Pre-drug phase to post-drug phase
1×10^{-3}M	1	Stop*	10
1×10^{-4}M	2	234	-2
	3	-14	-26
	4	2	-30
5×10^{-4}M	5	35	8
5×10^{-5}M	6	-24	-26
	7	-19	-23
	8	-17	-37
1×10^{-5}M	9	-2	-2
1×10^{-6}M	10	13	31
1×10^{-7}M	11	-4	-11

*Stop indicates that no mating calling pattern could be elicited.

Table 35. Per cent change in total pulses per vocal phase during carbamylcholine chloride experiments

Drug concn	Exp no.	Pre-drug phase to drug phase	Pre-drug phase to post-drug phase
1×10^{-3}M	1	Stop*	25
1×10^{-4}M	2	226	-6
	3	-16	-28
	4	-2	-32
5×10^{-4}M	5	39	15
5×10^{-5}M	6	-25	-28
	7	-15	-24
	8	39	-23
1×10^{-5}M	9	-2	-1
1×10^{-6}M	10	12	34
1×10^{-7}M	11	-1	-7

*Stop indicates that no mating calling pattern could be elicited.

Table 36. Per cent change in stimulus-response latency during carbamylcholine chloride experiments

Drug concn	Exp no.	Pre-drug phase to drug phase	Pre-drug phase to post-drug phase
1×10^{-3}M	1	Stop*	10
1×10^{-4}M	2	12	-29
	3	-3	-12
	4	-5	-6
5×10^{-4}M	5	-49	-61
5×10^{-5}M	6	25	-12
	7	-16	-62
	8	81	115
1×10^{-5}M	9	18	29
1×10^{-6}M	10	-29	6
1×10^{-7}M	11	-19	-31

*Stop indicates that no mating calling pattern could be elicited.

sympathetic nerve terminals. Norepinephrine is also released at nerve terminals in the central nervous system.

l-Norepinephrine bitartrate (Sigma) was given to seven isolated brainstem preparations in concentrations ranging from 1 x 10^{-3}M to 1 x 10^{-6}M (Tables 37-40). At the highest concentration of 1 x 10^{-3}M electrically evoked mating calling activity was reversibly blocked during the drug phase in two of the three preparations. The initial pulse rate was decreased or unchanged during the drug phase of the five preparations in which calling activity was not blocked. The effects of norepinephrine on the other three variables was inconsistent in the direction of change.

10. Gamma-aminobutyric acid (GABA)

Gamma-aminobutyric acid (GABA) is believed to be the major inhibitory transmitter in the vertebrate brain; however, little is known about GABA pathways (reviewed by DeFeudis, 1975).

GABA (Sigma) was given to six isolated brainstem preparations. It reversibly stopped electrically evoked mating calling during the drug phase in one preparation at a concentration of 5 x 10^{-3}M and in three preparations at a concentration of 1 x 10^{-3}M. It did not stop calling activity in two preparations at a concentration of 1 x 10^{-4}M.

Table 37. Per cent change in initial pulse rate during norepinephrine bitartrate experiments

Drug concn	Exp no.	Pre-drug phase to drug phase	Pre-drug phase to post-drug phase
1×10^{-3}M	1	Stop*	1
	2	Stop	-4
	3	-10	-7
1×10^{-4}M	4	-2	-11
	5	-7	-6
1×10^{-5}M	6	0	0
1×10^{-6}M	7	0	-1

*Stop indicates that no mating calling pattern could be elicited.

Table 38. Per cent change in vocal phase duration during norepinephrine bitartrate experiments

Drug concn	Exp no.	Pre-drug phase to drug phase	Pre-drug phase to post-drug phase
1×10^{-3}M	1	Stop*	-50
	2	Stop	-64
	3	8	15
1×10^{-4}M	4	-31	5
	5	-8	14
1×10^{-5}M	6	10	-8
1×10^{-6}M	7	3	13

*Stop indicates that no mating calling pattern could be elicited.

Table 39. Per cent change in total pulses per vocal phase during norepinephrine bitartrate experiments

Drug concn	Exp no.	Pre-drug phase to drug phase	Pre-drug phase to post-drug phase
1×10^{-3}M	1	Stop*	-45
	2	Stop	-62
	3	-2	9
1×10^{-4}M	4	-31	5
	5	-8	14
1×10^{-5}M	6	10	-8
1×10^{-6}M	7	3	13

*Stop indicates that no mating calling pattern could be elicited.

Table 40. Per cent change in stimulus-response latency during norepinephrine bitartrate experiments

Drug concn	Exp no.	Pre-drug phase to drug phase	Pre-drug phase to post-drug phase
1×10^{-3}M	1	Stop*	149
	2	Stop	4
	3	16	-30
1×10^{-4}M	4	1	-30
	5	-2	4
1×10^{-5}M	6	30	111
1×10^{-6}M	7	20	54

*Stop indicates that no mating calling pattern could be elicited.

11. Other drugs

In like manner, other drugs were also surveyed. These drugs were dopamine hydrochloride, l-epinephrine bitartrate, glycine, 5-hydroxytryptamine creatinine sulfate complex, methylsergide maleate, picrotoxin, pilocarpine nitrate, and strychnine sulfate. No dramatic changes in the pattern of electrically evoked mating calling activity occurred after a few experiments with each drug.

D. Discussion

1. Sham drug studies

The sham drug experiments demonstrated the degree of change in four variables of the neural correlates of mating calling activity that can occur in the isolated brainstem preparation in the absence of any drug. The initial pulse rate was observed to be the most stable variable with a mean absolute change well below that of the other three variables (Tables 5 and 6).

The degree of variability in vocal phase duration and total pulses per vocal phase that occurred in the sham drug studies may reflect the degree of variability occurring naturally in frogs. The vocal phase duration and total pulses per vocal phase are the correlates of the call duration and total pulses per call of the mating

calls of intact frogs. The mating call of R. p. pipiens shows considerable variability in call duration. The mating call has been described as lasting from over one second to over five seconds. The call usually is at least two or three seconds long. Some, but not all, of this variability in call duration is due to temperature. The number of pulses per call also varies from approximately 20 to 50 pulses (Pace, 1974). The total pulses per call do not vary with temperature.

The initial pulse rate of neural correlates of electrically evoked mating calling showed little variability in the sham drug experiments. This may also reflect the normal stability of pulse rates naturally occurring in mating calls at a given temperature. Data suggest that the pulse rate is a major physical basis by which gravid females recognize mating calls of their own species. This was shown when breeding females of two sympatric species of hylids, Hyla ewingi and H. verreauxi, discriminated between two synthetic signals that differed in pulse rates. The females were attracted by the signal with the pulse rate corresponding to that of their own species' mating call (Loftus-Hills and Littlejohn, 1971).

2. Drug studies

Preliminary drug studies measuring the neural correlates evoked during the pre-drug, drug, and post-drug

phases failed to yield positive results. The changes that occurred in the generation of the mating calling pattern were inconclusive. None of the drugs tested yielded graded, dose-related changes in initial pulse rate, vocal phase duration, total pulses per vocal phase, or stimulus-response latency. In most cases, even the direction of change in the variable was inconsistent.

The only consistent repeatable drug effect demonstrated was the ability of drugs, at experimentally determined concentrations, to stop electrically evoked mating calling activity during the drug phase. This was demonstrated by median effective concentration determinations (ED50) performed on eserine salicylate, atropine sulfate, and d-tubocurarine chloride. The ED50 and standard deviation for each drug was:

Eserine salicylate.....$5.90 \times 10^{-4}M \pm 2.39 \times 10^{-4}M$

Atropine sulfate.....$3.98 \times 10^{-4}M \pm 0.19 \times 10^{-4}M$

d-Tubocurarine chloride..$1.80 \times 10^{-5}M \pm 3.54 \times 10^{-5}M$

These ED50 determinations showed that the three drugs were capable of preventing electrically evoked mating calling activity when applied to the entire isolated frog brainstem. The ED50 also showed that the drug effect was reversible by rinsing the drug from the tissue bath and demonstrated that the ability of the three drugs to stop calling activity was dose-related to the extent that higher drug concentrations were more likely to stop

electrically evoked mating calling activity than were lower drug concentrations.

3. Feasibility

When this research project was conceived, it was hoped that drugs would be found that cause consistent, repeatable changes in the generation of the mating calling pattern. Finding these drugs may have given clues as to the central mechanism of calling. The isolated brainstem technique has several features that could be advantageous in a drug study. These include:

1) it provides a method for studying the central generation of mating calling activity at the level of the central nervous system,
2) it brings the onset of mating calling activity under the control of the investigator,
3) it completely eliminates any peripheral involvement in the generation of the calling pattern,
4) it insures that the effects of drugs will be on the central nervous system and will not be complicated by peripheral involvement,
5) it provides easy access to the calling circuits for performing experimental procedures,
6) it allows mating calling activity to be monitored from a single nerve, and
7) the isolated brainstem preparation and the continuous

flow-through tissue bath provide control over the brainstem's milieu, allowing for rapid changes of saline solutions without mechanical disturbance to the preparation.

Despite these advantages, the isolated brainstem technique's use in drug studies was found to be unsatisfactory. A major problem with the drug study was that drugs were applied to an entire brainstem in an effort to change the central generation of a complex yet specific behavior pattern. The drugs studied have not been shown to act selectively on specific behaviors but rather have more generalized effects throughout the nervous system.

Other disadvantages of the isolated brainstem technique for drug studies of neural correlates of mating calling are:

1) the surgical preparation of the isolated frog brainstem is difficult, requiring a high degree of manual dexterity,
2) the preparation of the isolated brainstem requires several hours, limiting the number of experiments that can be done,
3) not all preparations are successful, i.e. acceptable mating calling activity cannot be electrically evoked in all preparations and not all preparations remain viable for the duration of the experiment,
4) effects of drugs may be irreversible or require long

rinsing times, thus limiting the number of times a drug can be given to a brainstem preparation,

5) variability in vocal phase duration, total pulses per vocal phase, and stimulus-response latency within the same preparation is so great that small drug effects may be masked,

6) neural correlates vary among different preparations,

7) tissue transmitter levels in the frog brain and the utilization of these tissue transmitters in the frog brain have been shown to vary with the seasons (Harri, 1972a, 1972b; Sauerbier, 1977, 1978); therefore seasonal variation in sensitivity to drugs is likely, and

8) numerous isolated brainstem preparations are needed to show that a drug effect exists, is repeatable, and is dose-related.

To continue to apply drugs indiscriminately to the isolated brainstem in an effort to find a drug or drugs that consistently alter mating calling activity in a graded, dose-related manner is not recommended. Due consideration should be given to the individual merits and probable results of any future drug studies.

CHAPTER VI

SUMMARY AND CONCLUSIONS

A. Restatement of objectives

The objectives of this research project were 1) to quantitatively describe the normal neural correlates of mating calling activity electrically evoked in the isolated brainstem and to provide a measure of their variability among different brainstem preparations, 2) to determine the effects of different temperatures on electrically evoked mating calling activity, and 3) to investigate the feasibility of using the isolated brainstem preparation in drug studies of mating calling activity.

B. Normal neural correlates

1. Summary

Neural correlates of electrically evoked mating calling activity were recorded from 42 isolated brainstem preparations. Five variables of neural correlates were quantitated and statistically analyzed. The data were grouped and graphically displayed in histograms. The values for vocal phase pulse rate and vocal phase duration were compared with the values for call pulse rate and call

duration reported in the literature for the four mating call types identified by Littlejohn and Oldham (1968).

Four of the five variables, i.e. initial pulse rate, vocal phase pulse rate, vocal phase duration, and total pulses per vocal phase were normally distributed. The data on stimulus-response latency were inconclusive.

Initial pulse rate and vocal phase pulse rate were the least variable from one preparation to the next. Stimulus-response latency was the most variable.

The neural correlates of electrically evoked mating calling activity of Rana pipiens pipiens closely resembled the temporal characteristics of mating calls of the Northern call type, the call type given by intact R. p. pipiens. The neural correlates did not resemble closely the temporal characteristics of mating calls of the other three call types.

2. Conclusion

Some of the species-specificity of the mating call of R. p. pipiens is retained and revealed in the temporal pattern of neural correlates of electrically evoked mating calling activity recorded from the isolated frog brainstem.

C. Effects of temperature

1. Summary

Neural correlates of electrically evoked mating

calling activity were recorded at every 0.5 C^{o} interval between 18.0^{o} and 24.0^{o}C in three isolated brainstem preparations. To determine the existence of a relationship between temperature and each variable studied, a correlation coefficient was determined for each of the five variables. The relationship that was found between temperature and four of these variables was then described by a linear regression line.

A highly significant ($p < 0.001$) positive correlation indicating a high degree of relationship was found to exist between temperature and initial pulse rate ($r = 0.848$) and between temperature and vocal phase pulse rate ($r = 0.897$). These relationships were defined by the linear regression lines $Y_c = -6.84 + 1.38X$ and $Y_c = -10.35 + 1.48X$, respectively. A highly significant ($p < 0.001$) negative correlation indicating a moderately high degree of relationship was found to exist between temperature and vocal phase duration ($r = -0.626$). This relationship was defined by the linear regression line $Y_c = 6.00 - 0.19X$.

Total pulses per vocal phase was not affected by temperature.

A significant ($p < 0.05$) negative correlation indicating a low degree of relationship existed between temperature and stimulus-response latency ($r = -0.392$).

2. Conclusions

Initial pulse rate and vocal phase pulse rate are directly proportional and vocal phase duration is inversely proportional to temperature.

The effects of temperature on electrically evoked mating calling in isolated brainstems suggested that the previously-reported effects of temperature on mating calls of intact frogs is due primarily to effects of temperature on the central nervous system.

D. Effects of drugs

1. Summary

Drug studies consisting of a pre-drug, a drug, and a post-drug phase were performed using drugs known to act on neural transmitter systems. Emphasis was placed on drugs affecting the cholinergic system. The drugs were dissolved in oxygenated saline and applied to the entire isolated brainstem during the drug phase. Neural correlates of electrically evoked mating calling activity recorded during each of the three phases were compared for indications of drug effects on the elaboration of the mating calling pattern. None of the drugs tested yielded graded, dose-related changes in any of the four variables studied.

The median effective concentration (ED50) of drug

that reversibly stopped mating calling activity during the drug phase was determined for atropine sulfate (4.0×10^{-4}M), d-tubocurarine chloride (1.8×10^{-5}M), and eserine salicylate (5.9×10^{-4}M). The results showed that some of the drugs studied, at experimentally determined concentrations, reversibly stopped electrically evoked mating calling activity in the isolated frog brainstem. Experiments with these same drugs at lower concentrations as well as with other drugs yielded inconclusive and often inconsistent results.

2. Conclusion

The drugs applied to the entire isolated brainstem failed to cause reproducible, dose-related changes in the variables measured. The ED50 determinations had limited value in studying drug effects on neural correlates. Therefore, applying drugs to the isolated brainstem in the manner used for this dissertation is not a feasible technique for future drug studies of the central generation of mating calling.

E. Suggestions for future research

1. Calling types within the Rana pipiens complex

The similarities in the temporal characteristics of neural correlates of electrically evoked mating calling

activity in the isolated brainstem of R. p. pipiens and mating calls of the Northern call type recorded in the field led to the hypothesis that the species-specificity of all four mating call types of Littlejohn and Oldham (1968) will be retained and reflected in neural correlates of mating calling in the isolated brainstem. Such a hypothesis could be tested by evaluation of the temporal characteristics of electrically evoked mating calling in the isolated brainstems of frogs giving the other mating call types. Proving the hypothesis would provide further evidence that fixed action (behavior) patterns are programmed in and controlled by the central nervous system.

2. Effects of temperature on mating calling

Additional research should be done to test the conclusion that initial pulse rate and vocal phase pulse rate are directly proportional to temperature and that vocal phase duration is inversely proportional to temperature. The results thus far were obtained from only three isolated brainstem preparations. Additional experiments should support further the conclusions reached in this dissertation and increase the predictive efficacy of the resulting linear regression lines. Future experiments could also extend the range of temperatures beyond the 18° to $24^{\circ}C$ range reported in this dissertation.

3. Computerized quantitation

Future research on normal neural correlates of mating calling and on the effects of temperature on neural correlates could look at other variables not examined in this dissertation such as pulse width, interpulse intervals, and the rate of decline in pulse rate during the vocal phase. Future research on these variables as well as the ones already examined would be facilitated greatly by recording the electrically evoked mating calling activity on magnetic tape and feeding it into a computer. A properly programmed computer would relieve the researcher of the manual quantitation of data (one of the most time-consuming aspects of this research) and would be more accurate in detecting small changes in calling patterns.

4. Drug study techniques

If further drug studies are done on electrically evoked mating calling using the isolated frog brainstem technique, better methods of drug application should be found. Two methods that have been considered (drug stimulation of the brain and microiontophoretic application of drugs) have serious drawbacks.

Drug stimulation of the brain is a technique for studying the central effects of drugs on systemic functions or behaviors by delivering the drugs to discrete brain

sites in hopes of localizing the drugs' actions. A discrete brain site would be a part of the brain constituting a separate functional or anatomical entity, such as a nucleus. Usually animals the size of rats or larger are used so that the desired site is large enough to be identified and so as to assure that the drug remains in the desired area. Even in the rat, an injection volume of 0.5 µl is the maximum that should be used so as not to affect too large of an area in the brain (Myers, 1974). The brain of R. p. pipiens is so small that it seems unlikely that the action of a locally applied drug can be limited to a discrete brain site with present drug stimulation techniques.

Microiontophoretic application techniques may result in application of drugs to such a small area of the frog's brain that it would not yield information of value to the understanding of the central generation of the mating calling pattern. Myers (1974) explained that

> Molecularly, (iontophoresis) is an isolated preparation. An iontophoretically-induced change in the firing pattern of a single neuron may tell much about that cell but reveal very little about the relationship of that neuron to others in the same substructure. More remote is the role of the stimulated cell in a chain of interconnecting neurons. As such, information on the relationship between that cell and its role in a systemic function or behavior is hard to derive.

Thus, microiontophoretic application of a drug to the isolated frog brainstem in an effort to effect a change in electrically evoked mating calling does not appear feasible.

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APPROVAL SHEET

The dissertation submitted by David Douglas Schaffer has been read and approved by the following committee:

Dr. Robert S. Schmidt, Director
Associate Professor, Pharmacology, Loyola

Dr. Anthony J. Castro
Associate Professor, Anatomy, Loyola

Dr. Alexander H. Friedman
Professor, Pharmacology, Loyola

Dr. Alexander G. Karczmar
Professor and Chairman, Pharmacology, Loyola

Dr. Mary D. Manteuffel
Associate Professor, Biochemistry, Loyola

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Date

Robert S. Schmidt
Director's Signature