Differentiated roles of the periaqueductal gray and the paralemniscal area on vocalization in the new world bat *Phyllostomus discolor*.

Thomas Fenzl

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Foreword

The main goal of this project was to obtain information at systemic level on differentiated neural control of communication calls on the one hand and echolocation calls on the other hand in the bat, as a mammalian animal model.

Why study neural control of vocalization in animals?

Vocal behavior can be subdivided into three levels of complexity. The most complex vocal behavior is represented by human speech. The next, less complex behavior would be vocal imitation, in which, besides initiation of a vocal pattern also the acoustic structure of the pattern is voluntarily controlled. This can be described as vocal plasticity (Jürgens, 2002). An example for vocal imitation is song learning in songbirds or the songs of whales. The lowest level of vocal behavior is represented by the genetically determined vocal reaction. Laughing or shrieking, crying or shouting in humans, i.e., the so called nonverbal emotional utterances, as well as monkey`s or bat`s calls for example belong to this group of vocal behavior. Since animal calls and nonverbal emotional utterances, and in addition emotional intonations during affective speaking most probably represent homologous vocal behaviors (Jürgens, 1998), animal models are important to study neural control mechanisms of this type of vocalization. New results could not only contribute to principle questions about the neural components of the vocal system but, could also help curing human disorders such as dysarthry or soothing the effects of a stroke which hit vocalization related brain areas.

I. Abstract

The interaction of neural components contributing to vocal behavior in mammals is far from being understood. Above that, ongoing research indicates that the role of neural components involved in the process of vocalization is not resolved in detail in many cases. One brain area turned out to play a crucial role on nonverbal vocal behavior, namely the periaqueductal gray matter (PAG). Research was done mostly in non-human primates, non-primate monkeys and cats. From bats there is evidence that the paralemniscal area (PLA) plays a similar crucial role for the control of echolocation calls as the periaqueductal gray does for communication calls.
Up to date the neural mechanisms for the production of communication calls and the production of echolocation calls have not been investigated together in a single animal. The neotropical bat, *Phyllostomus discolor* with its rich repertoire of communication calls and its ability to echolocate lends itself to such a combined study of periaqueductal and paralemniscal control of vocalizations.

Electrical microstimulation elicits several types of communication calls, as well as echolocation calls at distinct regions within the periaqueductal gray of the bat. Both classes of calls are not distinguishable from spontaneously emitted calls. Microdialysed kainic acid (GLU agonist) into this regions demonstrates that activity of neurons and not fibers of passage are responsible for the vocal responses. Respiration is generally synchronized with electrically and pharmacologically induced vocalizations.
This indicates that the periaqueductal gray is involved in vocal pathways for the control of both communication calls and echolocation calls.

In *Phyllostomus discolor*, the paralemniscal area has similar properties as found in other bats. Echolocation calls which resemble to natural echolocation calls can be elicited in a sharply delimited area with electrical microstimulation at very low thresholds. Communication calls can not be triggered in the paralemniscal area. The activated elements in the paralemniscal area are again the neurons, not fibers of passage as demonstrated with microdialysed kainic acid.

The paralemniscal area seems to be involved in the control of echolocation calls, exclusively.

Elicitability of communication calls and echolocation calls via chronically implanted microstimulation electrodes into the periaqueductal gray is differently affected by kynurenic acid (GLU antagonist), which was simultaneously applied for reversible inactivation of the paralemniscal area. When applied iontophoretically into the contralateral paralemniscal area periaqueductally triggered echolocation calls are selectively and reversibly blocked, whereas periaqueductally triggered communication calls remain unaffected. Ipsilateral application of kynurenic acid has no effect on neither communication calls nor echolocation calls triggered in the periaqueductal gray. *The results indicate that echolocation calls and communication calls must be controlled via at least partly separated vocal pathways below the level of the periaqueductal gray.*

Tracer injections of WGA-HRP into vocally active sites within the periaqueductal gray give rise to projections towards the region of the nucleus ambiguus/retroambiguus complex (NA/NRA-complex). The nucleus ambiguus in this bat could be identified by AChE-staining.

These preliminary data could support a direct PAG-NRA pathway in Phyllostomus discolor as one implementation of vocal control. Other control pathways for vocalization must exist as the results on the production of echolocation calls and its suppression by PLA blockades suggest. However, no direct PAG-PLA projection could be demonstrated to date.

II. Zusammenfassung

Das Zusammenspiel neuronaler Komponenten, die zu vokalem Verhalten beisteuern, ist bei weitem nicht verstanden. Darüber hinaus verdeutlicht die aktuelle Forschung, dass in vielen Fällen nicht einmal die Rolle neuronaler Bereiche, beteiligt am Prozess der Vokalisation, im Detail geklärt ist. Ein Gebiet des zentralen Nervensystems, das zentrale Höhlengrau (PAG), nimmt jedoch, und das zeigte sich bei zahlreichen Untersuchungen, eine zentrale Rolle bei nichtverbalen vokalem Verhalten ein. Forschung hierzu wurde vor allem an Primaten, niederen Affen und Katzen durchgeführt. Bei Fledermäusen gibt es Hinweise, dass dem paralemniscalen Gebiet (PLA) eine ähnlich wichtige Rolle bei der Kontrolle von Ortungslauten zukommt, wie die des zentralen Höhlengraus bei der Kontrolle von Kommunikationslauten.

Elektrische Mikrostimulation löst in abgrenzbaren Bereichen des zentralen Höhlengraus der Fledermaus unterschiedliche Kommunikationslaute und Ortungslaute aus. Beide Lautklassen sind nicht von spontan geäußerten Lauten zu unterscheiden. Durch Mikrodialyse in diese vokal aktiven Bereiche appliziertes Kainat (GLU Agonist) belegt die Beteiligung von Neuronen und nicht von vorbeiziehenden Faserbündeln an vokalen Antworten. Die Atmung ist generell mit elektrisch als auch pharmakologisch induzierten Vokalisationen synchronisiert.
Dies zeigt, dass das zentrale Höhlengrau in vokale Pfade eingebunden ist, welche sowohl Kommunikationslaute als auch Ortungslaute kontrollieren.

Das paralemniscale Gebiet hat in *Phyllostomus discolor* Eigenschaften, wie sie ähnlich auch bei anderen Fledermäusen gefunden werden. Ortungsrufe, die natürlichen Rufen entsprechen, können mit elektrischer Mikrostimulation unter Verwendung sehr geringer Reizströme in einem scharf abgegrenztem Gebiet ausgelöst werden. Kommunikationslaute hingegen können in diesem Gebiet nicht ausgelöst werden. Hierbei handelt es sich wiederum um Neurone und nicht um vorbeiziehende Faserbündel, wie sich durch Applikation von Kainat zeigt.
Das paralemniscale Gebiet scheint ausschließlich an der Kontrolle von Ortungslauten beteiligt zu sein.

Die Auslösbarkeit von Kommunikationslauten und Ortungslauten über chronisch in das zentrale Höhlengrau implantierte Reizelektroden wird durch Kynurensäure (GLU Antagonist), das zur reversiblen Blockade des paralemniscalen Gebietes simultan appliziert wird, unterschiedlich beeinflusst. Ortungslaute, ausgelöst im zentralen Höhlengrau, können selektiv und reversibel durch Mikrodialyse des Antagonisten im kontralteralen paralemniscalen Bereich blockiert werden. Kommunikationslaute, die ebenfalls im zentralen Höhlengrau induziert werden, lassen sich durch paralemnsicale Blockaden nicht unterbinden. Ipsilaterale Blockaden haben weder eine Auswirkung auf die Erzeugung von Kommunikationslauten, noch auf die Erzeugung von Ortungslauten.
Diese Befunde deuten darauf hin, dass Ortungslaute und Kommunikationslaute zumindest durch teilweise separierte vokale Pfade unterhalb der Ebene des zentralen Höhlengraus kontrolliert werden müssen.

In vokal aktive Bereiche des zentralen Höhlengraus applizierte Tracer (WGA-HRP) zeigen Projektionen in Richtung des Nucleus ambiguus/retroambiguus Komplexes (NA/NRA-Komplex) auf. Der Nucleus ambiguus konnte in *Phyllostomus discolor* durch AChE-Färbung identifiziert werden.
Diese vorläufigen Daten könnten einen direkten PAG-NRA Pfad zur Verwirklichung vokaler Kontrolle bei Phyllostomus discolor implementieren. Wie die Ergebnisse über die Erzeugung von Ortungslauten und deren Unterdrückung durch PLA-Blockaden zeigen müssen weitere vokale Pfade existieren. Bis jetzt konnte jedoch keine direkte PAG-PLA Projektion aufgezeigt werden.

III. Introduction

1. Communication systems

Behavioral interactions, used by the transmitter to influence the behavior of the receiver require effective communication systems. To do so, animals make use of several channels for sensory communication. Pheromones used as a chemical signal are probably the oldest means of communication. The aggregation of *Dictyostelium* for example, an amoeba with a partly social life cycle is controlled through species-specific pheromones. In short range interactions, touch is another form of communication. Grooming in monkeys not only helps to control ectoparasites but also commits individuals to a social group. In courtship-bound communication, touch is used in invertebrata as well as vertebrata in a similar manner. Visual signals, e.g. color patterns of the skin and postures of the body or body parts are wide spread communicative means. The most adaptive communication system however is represented by vocal behavior. Here the auditory signals can serve in short, medium or long range communication, either via airborne sound waves in terrestrial animals, water-borne sounds in aquatic animals such as some pisces and cetacea or via substrate vibrations produced by some terrestrial animals.

1.1 Vocal communication – different levels of complexity

Recognizing sound requires detecting waves of alternating pressure, with wave length ranging upwards from about 20 Hz, lower frequencies are usually felt as vibrations and not perceived as sound (Barnes et al., 1991). The pressure of the sound wave gives the loudness, proportional to the amplitude while the sound`s pitch is determined by the frequency. By modifying these parameters vocal structures become the vocal repertoire of an animal and can serve as a vocal communication system.

This communication system, represented in vocal behavior is organized at three different levels of complexity:

The lowest level of vocal behavior is represented by a completely genetically determined vocal reaction. A strong grasp at a monkey`s limb generates a stereotyped vocal reaction and a heavy blow against the body of an infant, for instance, elicits shrieking from birth on. The infant's shrieking reaction and the monkey call may be considered as a reflex behavior (Jürgens, 2002).

Another, higher-level vocal behavior is vocal imitation as observed in songs performed by whales (Payne and Payne, 1985). In this case, the initiation process of an (innate) vocal pattern, as well as the acoustic structure of the pattern are voluntarily controlled meaning that there is vocal plasticity (Jürgens, 2002).

The most complex level of vocal behavior is represented in human speech. There is not only voluntary control of initiation and of acoustic structure of the vocal utterances, but also attribution of specific meaning to these utterances (Jürgens, 2002).

1.2 The special position of speech

Although the ability for acoustic communication is present throughout the class of vertebrata (and invertebrata in a broader sense) the ability to speak is shared with no other living creature, it is species-specific to *Homo sapiens* (Ploog, 1988). This basic differentiation in vocal communication, on the one hand human speech, on the other hand vocal imitation and genetically determined vocal reaction can not only be linked to particular behavioral aspects but can also be traced back to differentiated neuroanatomical (pre-)adaptations. The central nervous control of monkey calls for example and the control of human speech, emphasizing the crucial role of cortical structures, differs in several aspects.

The supplementary motor area and the anterior cingulate gyrus are both vocally active on electrical microstimulation. But only in humans microstimulation of the supplementary motor area leads to the utterance of vocalizations while stimulation within the anterior cingulate gyrus elicits vocalizations only in non-human mammals and not in humans (Erickson & Woolsey, 1951; Jürgens & Ploog, 1970). As a consequence to findings from

several other authors Jürgens (2002) suggests that the anterior cingulate gyrus is involved in volitional control of emotional states and that of innate motor patterns, while the supplementary motor area is involved in the volitional control of learned motor patterns [speech]. Both areas have in common that they control the initiation rather than the pattern generation of vocal utterances.

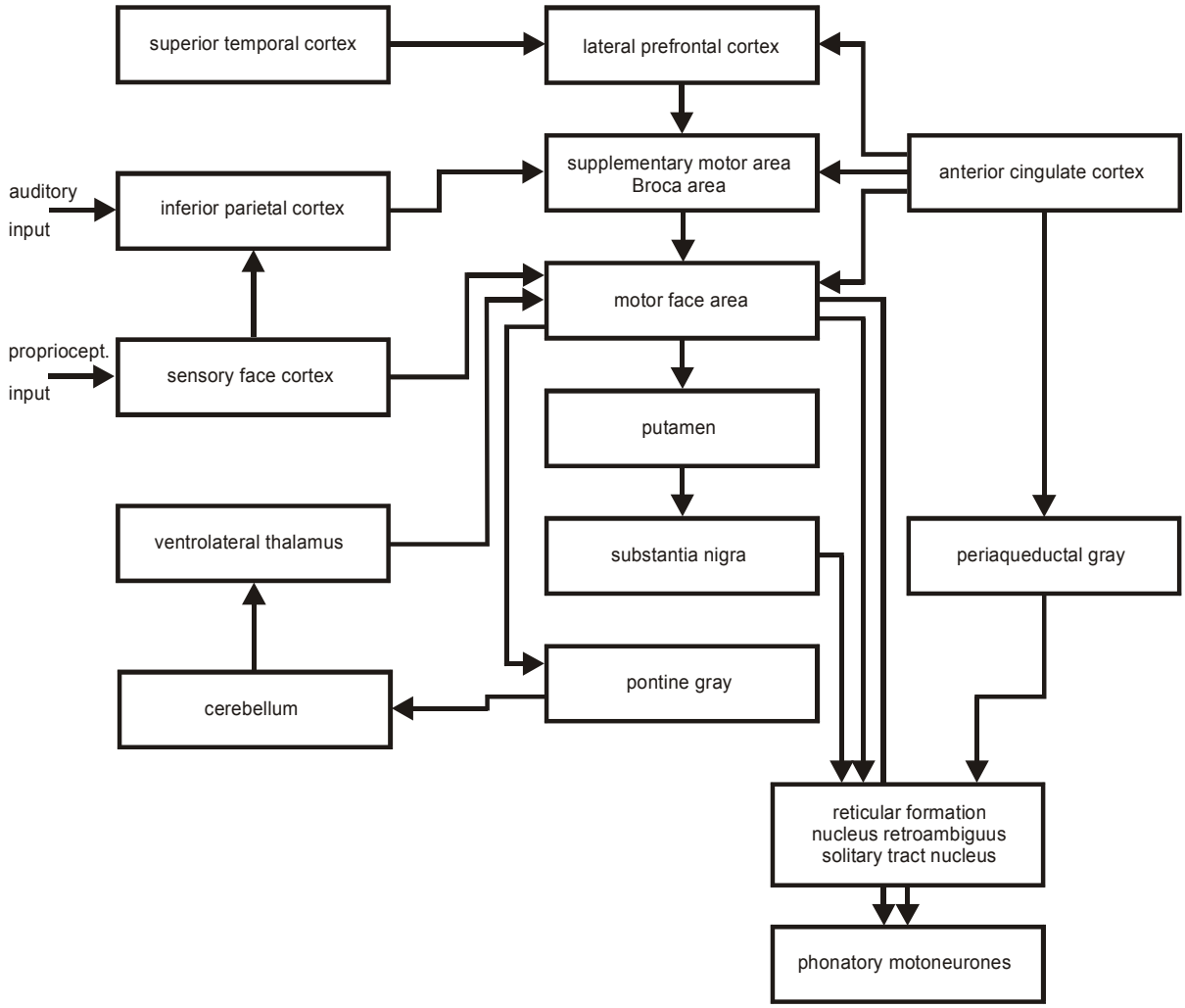

FIG. 1: Central control of speech production.
The reticular formation, the nucleus retroambiguus and the nucleus of the solitary tract represent the lowest level of this hierarchically organized control system of vocal activity. At this level, the network integrates laryngeal, respiratory and articulatory activity using direct access to phonatory motoneurons. Input to this level of control follows two main streams. One main input originates from the cortico-medullary descending motor pathways to transmit information from the motor cortex for motor

coordination of learned motor patterns. The other input probably exerts a gating function controlling the release of vocal utterances and has its origin in the periaqueductal gray. Arrows indicate direct anatomic connections, structures within a box are directly connected. Adapted, from Jürgens (2002).

Another difference in neural organization can be found in the direct motor pathway from the laryngeal representation in the primary motor cortex to the laryngeal motoneurons in the medulla (FIG. 1). Cortico-spinal pathways, intimately associated with the motor systems of the spinal cord for the control of voluntary movements, are not only represented in humans. However the face area in humans for example, which is used in tasks demanding great precision and fine control, is represented disproportionately large in the motor area of the cortex when compared with the area in the monkey. The above mentioned laryngeal projection is realized in man only (Jürgens & Ploog, 1976; Kuypers, 1958) representing the neuronal base for the voluntary control of the vocal folds. While man is able to produce a wide variety of acoustic patterns at will, vocal operant conditioning experiments in macaques have shown that acoustic structures of their calls can only be shaped in a very limited way (Sutton et al, 1973). Although monkeys clearly have some voluntary control on the initiation of species-specific calls, they can control the frequency and amplitude modulation only to a very limited extend (Jürgens, 1988).

The vocal folds usually vibrate during vocalizations, this is true for animals as well as for humans. But in speech, and this is a fundamental difference to vocalizations produced by animals, articulation is used to produce different speech sounds such as vowels and consonants. This is achieved by shaping the vocal tract (parts of the human vocal organs above the level of the larynx) with movements of the soft palate, tongue, lips and jaw, collectively called the articulators.

The face area within the motor cortex controls these movements used for articulation. Electrical microstimulation produces movements of vocal folds, tongue, jaw and lips (Jürgens, 2002). If this area is lesioned bilaterally in humans, a syndrome called pseudobulbar palsy can be observed, expressed by complete loss of voluntary control over the speech apparatus (Groswasser er al., 1988) used for articulation, while in nonhuman primates bilateral distortion has no effects on the production of species-specific calls (Jürgens et al, 1982).

BOX 1: Origins of speech

" With the domination over Nature beginning with labor… with every new advance toward the human horizon… the evolving human beings came to the point where they had something to say to each other. The necessity created the organ: The undeveloped larynx of the apes transformed itself slowly but surely through modulation by steadily increasing modulation, and the organ of the mouth gradually learned to pronounce one articulated letter [sic] after another"
(Engels, F.; 1876; taken from Hewes, 1977)

Friedrich Engels had discussed the role of labor in the "hominization" of apes in 1876 and had placed the origin of articulate speech in the lower status of savagery (Hewes, 1977). Social writers like Engels, who found in the linkage of cooperative labor and language a congenial theory somehow glorifying the working man, based their discussions on Noirè**,** who wrote a thoughtful treatise on the role of tools and tool making in human evolution in 1880 (Hewes, 1977). The late 19th and early 20th post-Darwinian century came up with a few more theories on the evolution of language. According to Hewes, in the "Ding-Dong Theory", which was much to the contribution of F. Max Müller, man had an innate propensity for associating certain sounds with certain kinds of objects and actions, responding to them in a manner analogous to the way an object resonates when struck (Hewes, 1977). Another interesting theory on the origins of language, as outlined by Hewes, was proposed by Donavan in his "Festal Origin" of language in 1893/95. Donavan saw the beginnings of language in dance, song, and related expressive sound making (Hewes, 1977).
Among several other theories the "Gestural Theory of the Origin of Language" (Hewes, 1973, Hewes, 1973) became very prominent. Hewes argues that man's originally uttered language must have been primarily gestural, carried on with hand and arm signals rather than with vocal sounds. This theory is supported by a distinguished line of scholars, who's work can be traced back from the presence towards the first half of the 18th century (Hewes, 1973). He also mentions that the gestural theory seems to be the most attractive and advanced theory among the many glottogonic hypotheses and it receives support from recent studies carried out on chimpanzees and other primates. Additional support comes from an recent essay, where findings from behavioral psychology and neuroscience were incorporated leading the author to the statement that language evolved from referential pointing which, when combined with mimed movement, leads to a language of gesture and from imitating the calls of animals (Place, 2000).

1.3 Vocalizations of animals and nonverbal human utterances – homologous behaviors

Nonverbal emotional utterances of humans, such as laughing, shouting, crying as well as emotional intonations superimposed on the verbal component during affective speaking bear a strong genetic component (Jürgens, 1988). Support for this genetical determination comes from findings that even children born deaf produce nonverbal emotional utterances (Eibl-Eibesfeldt, 1973) and emotional vocal expressions are almost identical all over the world (Beier & Zautra, 1972). This stands in contrast to human speech with its learned motor patterns, resulting in an immense variety of languages.
Animal models are of limited use to study central nervous processes of speech production. But this is not true for human utterances. Kasper-Hauser studies in squirrel monkeys (Winter et al., 1973) have demonstrated that monkeys do not need to learn species-specific vocalizations from conspecifics (Jürgens, 2002), the acoustic structure of individual call types is genetically determined. With the assumption that nonhuman vocalizations (e.g. monkey calls) and nonverbal emotional utterances (human vocalizations) together with emotional intonations may be considered as homologous behaviors, it might be suitable to consider monkey calls as an appropriate model for investigations on the central mechanisms underlying emotional vocal expression (Jürgens, 1998).

2. On the search of vocalization-eliciting substrates

Exploration of vocally active sites using electrical brain stimulation can be traced back into the beginning of the 20th century: In the chimpanzee by Brown (1915) and others, in the cat by Gibbs and Gibbs (1936) and others, in the rhesus monkey by Magoun and coworkers (1937) and others and in man by Brickner (1940) and others. After world war II research was carried on in birds by von Holst and von Saint Paul (1960) and others, in frogs by Schmidt (1966) and others, in the squirrel monkey by Jürgens and coworkers (1967) and others and in lizards by Kennedy (1975). But it took until 1967 that Robinson (1967) made a more systematic study on the elicitability of vocalizations in the brain of

Macaca mulatta using electrical stimulation. Jürgens and Ploog carried out another very detailed and systematic brain-stimulation study in which the entire brain of the squirrel monkey was explored for sites where electrical microstimulation yield vocal responses (Jürgens & Ploog, 1970). They found that the vocalization-eliciting substrate was not restricted to a small discrete area but rather occupied a widely branching system, reaching from the forebrain down to the lower brainstem (FIG. 2).

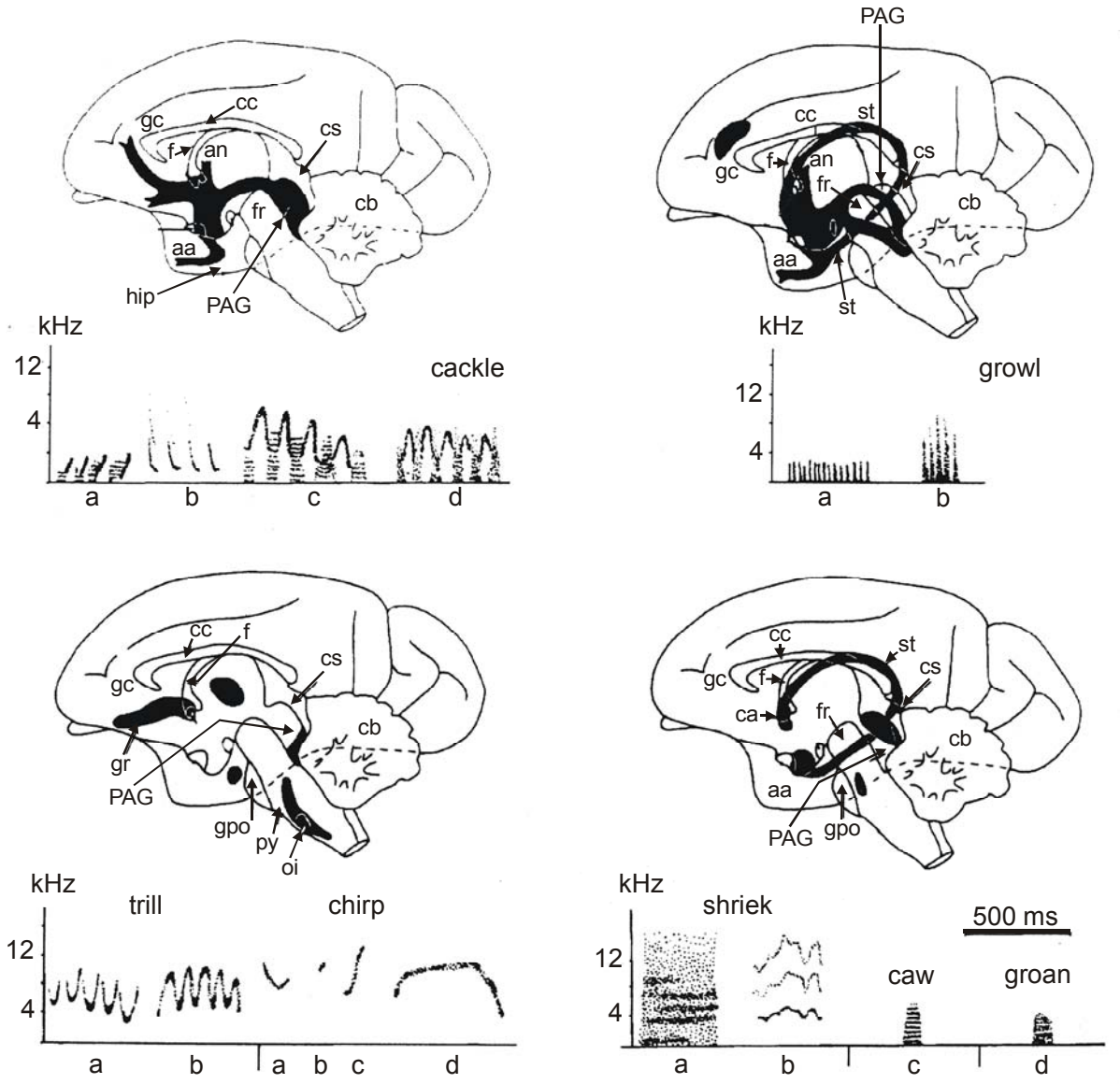

FIG. 2: The neural substrate for vocalizations in the squirrel monkey.
The vocally active sites are not organized in few delimited vocalization centers but rather are wide spread reaching from the forebrain to the lower brainstem. However, the majority of the active sites belong to the

limbic system. The calls, represented as spectrograms (a-d) below each drawing can be elicited in the black areas of the drawings. It can be seen that different types of calls are linked to different brain areas, with the exception of the periaqueductal gray in which virtually all call types can be triggered. *Abbreviations*: aa, anterior amygdala; an, nucleus anterior thalami; ca, commissura anterior; cb, cerebellum; cc, corpus callosum; cs, colliculus superior; f, fornix; fr, formatio reticularis; gc, cingulate gyrus; gpo, griseum pontis; gr, gyrus rectus, hip, hippocampus; oi, inferior olive; PAG, periaqueductal gray; py, tractus corticospinalis; st, stria terminalis. Adapted, from Jürgens (1998).

According to the authors, the majority of the vocalization-eliciting structures was found within the limbic system (see also BOX II, this page): septum, nucleus acumbens, preoptic area, hypothalamus, midline thalamus, amygdala and the bed nucleus of the stria terminalis, where different call types could be elicited. Only within the periaqueductal gray of the midbrain (see also BOX III, page 19) all types of calls could be triggered (Jürgens & Ploog, 1970).

BOX II: What is the limbic system? (findings in humans)

The majority of the structures which belong to the limbic system are paleocortical and archicortical. The notion was first created to describe areas covering the corpus callosum, nucleus caudatus, putamen, nucleus subthalamicus and substantia nigra (basal ganglia) and the diencephalon like a “limbus”. The modern anatomy describes a few more areas for the limbic system: hippocampus with fornix, gyrus cinguli, gyrus parahippocampalis with regio entorhinalis, gyrus dentalis, gyrus dentatus, corpus amygdaloideum, corpus mamillare. The definition of the limbic system represents neither a functionally combined central region nor topographically linked areas within the brain. The classic and still widespread assumption that the limbic system serves as a center for “self-maintenance” and reproduction is insufficient. Although the limbic system influences the “self maintenance” by controlling psychic parameters as well as reproduction through modulation of sexual and vegetative parameters, it is incorporated in numerous emotional, intellectual and instinct-bound performances without being misunderstood as the one and only origin of these abilities. Interestingly, vocalizations elicited within the anterior cingulate cortex in squirrel monkeys can be abolished by procaine and kynurenic acid injections into the PAG, leading Jürgens to the statement that there exists a limbic vocal control pathway at midbrain level from the cingulate cortex via the PAG for nonverbal emotional vocal utterances (Jürgens & Zwirner, 1996).

2.1 Vocalizations as primary and secondary reactions to stimuli

The limbic vocalization-eliciting sites are characterized by relatively long latencies of vocal responses and fast habituation to repetitive stimulation. This suggests that the elicited vocalizations are rather secondary reactions to stimulus-induced motivational changes than direct motor responses (Jürgens, 1998). This hypothesis was tested by electrical self-stimulation experiments. It was assumed that there is a high probability that electrically-induced motivational changes strong enough to induce vocalization as a secondary reaction do have positive or negative reinforcing qualities (Jürgens, 1976). An increase in motivation without the possibility of performing the adequate consummatory act (no goal object existing) would be negatively reinforcing, a decrease in motivation (drive reduction) would be positively reinforcing. The self-stimulation experiments in squirrel monkeys showed two types of vocalization-producing brain areas, i.e. those where electrically induced vocalizations were independent of the accompanying reinforcement and brain areas where vocalizations and reinforcement were correlated. The first group included the anterior cingulate gyrus, the adjacent supplementary motor area, gyrus rectus, ventro-medial edge of the capsula interna and the caudal periaqueductal gray with the adjacent parabrachial region. The second group included the caudatum, septum, substantia innominata, amygdala, inferior thalamic peduncle, stria terminalis, midline thalamus, ventral and periventricular hypothalamus, substantia nigra, rostral periaqueductal gray, dorso-lateral midbrain tegmentum and lateral medulla.

The author interpreted vocalization areas which did not show a correlation between the elicited call and accompanying reinforcement as primary vocalization areas and areas which show such a correlation as secondary vocalization areas. The anterior cingulate gyrus with a dorsal extension into the supplementary motor area and the caudal periaqueductal gray including the adjacent parabrachial region fulfilled best the criteria for primary vocalization areas (Jürgens, 1976).

2.2 The anterior cingulate cortex – its role in vocalization

Electrical stimulation of the anterior cingulate cortex produces vocalizations in non-human mammals such as squirrel monkey (Jürgens & Ploog, 1970) but not in man. In a vocal operant conditioning study in macaques, in which the animals had to perform a specific call in order to obtain food, the animals could no longer execute the task after bilateral removal of the anterior cingulate cortex (Sutton et al., 1974). Interestingly this operation did not interfere with a slightly altered conditioning task where the animals had to activate a lever without vocalizing to receive food. The involvement of the anterior cingulate cortex in the performance of such volitionally controlled vocalizations is supported by electrophysiological studies. Recording of field potentials in the anterior cingulate cortex was only possible while monkeys vocalized as an operant response to obtain food while vocal protest reaction after withdrawal of the food could not be connected with activity in this cortical area (Gemba et al., 1995). Single-unit recording experiments in macaques yield activity changes of neurons in this area during vocalization in a vocal operant conditioning experiment (West & Larson, 1995).
The anterior cingulate cortex seems to be involved in voluntary initiation and suppression of emotional utterances (Jürgens, 2002), at least in monkeys, although the role of this area does not seem to be limited to nonhuman primates (Jürgens, 1998). After a bilateral lesion in the anterior cingulate cortex due to occlusion of the ascending branches of both anterior cerebral arteries a human patient showed akinetic mutism. This patient recovered to normal with respect to speech (articulation, phonation, semantics and grammar) but remained deficient in emotional intonation (Jürgens & von Cramon, 1982; Jürgens, 1998).

2.3 The periaqueductal gray

The caudal periaqueductal gray has been probed in different species and demonstrated that vocalizations can be triggered within these regions: fish (Magoun et al., 1937), cat (Hunsberger, 1956), bird (Brown, 1971; Delius, 1965), frog (Demski & Gerald, 1974; Schmidt, 1966), lizard (Kennedy, 1975), squirrel monkey (Jürgens, 1979; Jürgens & Lu,

1993), rhesus monkey (Larson & Kistler, 1984), rat (Yajima et al., 1980), guinea pig (Martin, 1976) and bat (Suga and Yajima, 1988; Valentine et al., 2002).
Reasons to attribute an exceptional role to the periaqueductal gray among the vocally-active brain structures for the control of vocalizations were:

- The shortest latencies for vocalizations are found in the periaqueductal gray.
- The highest number of different vocalization types can be elicited in the periaqueductal gray.
- All vocalizations persist until the end of stimulation without habituation to the stimulus.
- There are several reports of transitory or even permanent mutism after periaqueductal lesions (Adametz & O´Leary, 1959; Hundsberger, 1956; Skultety, 1965).

Mammalian vocalizations in general incorporate a precise coordination of respiratory and laryngeal movements. The motoneurons responsible for control of respiratory movements are located in the anterior horn of the cervical, thoracic and upper lumbar spinal cord, those controlling the laryngeal muscles are found in the nucleus ambiguus and neurons responsible for the control of articulatory movements are localized in the trigeminal motor nucleus, facial nucleus, rostral nucleus ambiguus, hypoglossal nucleus and anterior horn of the upper cervical spinal cord (Jürgens & Ploog, 1981). At midbrain level, transection experiments in cats and monkeys have shown that animals become mute by lesions including the periaqueductal gray and the adjacent tegmentum (Jürgens & Ploog, 1981) while destruction of other midbrain structures had no effect on vocalization.

2.3.1 The vocalization controlling system is organized hierarchically

Further findings suggested a hierarchically organization of the vocalization controlling system including the periaqueductal gray. Vocalizations elicited from the periaqueductal gray where not affected by bilateral lesions in vocalization-eliciting areas rostral to it, but where abolished by lesions in the dorso-lateral pons and the ventro-lateral medulla (Jürgens, 1979).

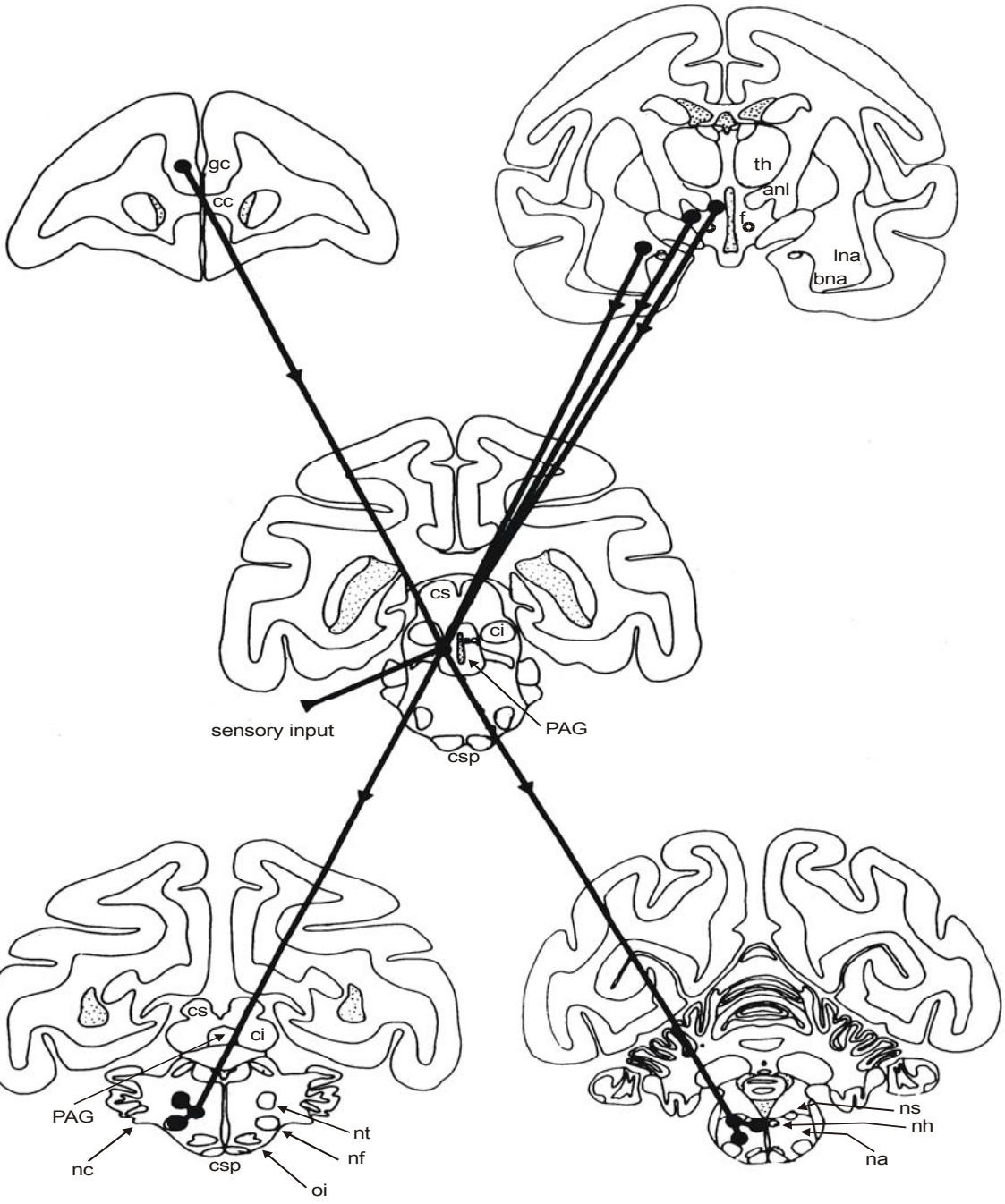

FIG. 3: Hierarchic central control of vocalizations in monkeys.
The anterior cingulate cortex represents the highest level of this organization. The periaqueductal gray receives input not only from this cortical site but also from motivation-controlling brain sites such as hypothalamus, amygdala and thalamus and from sensory pathways. The level of motor coordination (lowest level) is represented by phonatory motoneurons and interneurons in the lower brainstem and the spinal cord. *Abbreviations*: anl, ansa lenticularis; PAG, periaqueductal gray; bna, nucleus basalis amygdalae; ci, colliculus inferior; cs, colliculus superior; csp, pyramidal tract; gc, cingulate gyrus; lna nucleus lateralis amygdalae; na, nucleus ambiguus, nc, nucleus cochlearis; nf, nucleus facialis; nh, nucleus hypoglossus, ns; nucleus solitarius; nt, motor nucleus of trigeminus; oi, inferior olive; th, thalamus. Adapted, from Jürgens (Jürgens, 1988).

The periaqueductal gray receives input from several regions: Visual input reaches the region from the deep layers of the superior colliculus (Meller & Dennis, 1986), acoustic input comes from the inferior colliculus (Jürgens & Ploog, 1981; Mantyh, 1982), visceral input comes from the solitary tract nuclei (Bandler & Tork, 1987) and somatosensory input comes from the dorsal horn of the spinal cord and the spinal trigeminal nucleus (Harmann, 1988). These inputs may induce vocalization rather directly through external stimuli, e.g. pain-shrieking as response to a noxious stimulus (Jürgens, 1991).

In addition to sensory input, there is input from motivation-controlling limbic structures such as the amygdala, septum, hypothalamus and midline thalamus serving probably to modulate the vocal reactivity to external stimuli according to prior experience and momentary emotional state (Jürgens, 1991; Jürgens & Ploog, 1981). Connections are also found between the anterior cingulate cortex representing the highest level of the vocalizing system and the periaqueductal gray (FIG. 3). While this cortex site is unnecessary for (non-spontaneous) vocal reactions, it is needed for the voluntary control of vocalization (Jürgens & Ploog, 1981).

On the output side, the periaqueductal gray vocalization center is connected to the reticular formation around the nucleus retroambiguus which itself is connected with all phonatory motor nuclei (Holstege, 1989; Jürgens & Pratt, 1979; Thoms & Jürgens, 1987). More specifically, the periambigual reticular formation projects to the trigeminal motor nucleus responsible for jaw control, to the facial nucleus responsible for lip movements, to the hypoglossal nucleus responsible for tongue movements, to the nucleus ambiguus controlling the vocal folds and to the thoracic and upper lumbar ventral horn containing the expiratory motoneurons (Jürgens, 1994).

A potential pathway where the periaqueductal gray might exert its vocalization control on lower brainstem regions was traced in a combined stimulation/lesion study (Jürgens & Pratt, 1979). Lesions capable of blocking periaqueductally elicited vocalization could be traced up from the caudo-lateral periaqueductal gray into the lateral tegmentum underneath the inferior colliculus, from here, along the medial edge of the lateral lemniscus into the ventro-lateral pons and from there into the periambigual reticular formation (Jürgens, 2002).

2.3.2 The periaqueductal gray as a pattern generator of vocal patterns

Two subdivisions of the reticular formation, namely the parvocellular and the central nuclei of the reticular formation are thought to be involved in vocal pattern generation (Dusterhoft et al, 2000) rather than the periaqueductal gray itself as outlined by other authors (Holstege, 1989; Larson, 1991). In his anatomical study, Holstege found, that a specific cell group of the caudal periaqueductal gray and of the tegmentum lateral to it projects bilaterally to the nucleus retroambiguus. Neurons in this nucleus projected to the motoneuronal cell groups innervating mouth-opening and perioral muscles as well as to motoneurons innervating the pharynx, soft palate, and tongue and probably to the larynx (Holstege, 1989). Single unit recordings in monkeys have revealed vocalization-correlated periaqueductal activity (Larson & Kistler, 1984) and the activity of some cells has been reported to be correlated with EMG activity of specific laryngeal muscles. Similar results where obtained by Zhang and coworkers (1994). They recorded electromyographic (EMG) changes in respiratory, lanryngeal (and therefore vocalizations) and oral muscles evoked by microinjections of D,L-homocysteic acid (excitatory amino acid agonist) injected into the periaqueductal gray. Different vocalizations/muscle patterns where linked to individual periaqueductal sites and they concluded that the periaqueductal gray contains topographically separable groups of neurons coordinating laryngeal, respiratory and oral muscle patterns. Organization of the periaqueductal gray represents muscle patterns rather than representation of individual muscles (Zhang et al., 1994).

These findings led to the assumption that motor coordination of vocalizations is located in the periaqueductal gray.

In contrast to this, stand results from Düsterhoft and coworkers (2000). They found that the majority of periaqueductal vocalization-related cells fired before, but not during vocalization, while only a few cells in the parvocellular nucleus and no cells in the central nucleus of the reticular formation acted this way (Düsterhoft et al., 2000). In the periaqueductal gray no cell was found that changed its discharge rate in rhythm of frequency modulation whereas changes in discharge rates where found in the parvocellular nucleus and the central nucleus of the reticular formation (Düsterhoft et al., 2000).

BOX III: **The midbrain periaqueductal gray matter (PAG)**

The term “periaqueductal” is used for the ventricular central gray around the midbrain aqueduct distinguishing it from the rostrally continuing periventricular gray matter surrounding the third ventricle in the hypothalamus and thalamus and from the caudally bordering periventricular gray matter which constitutes the ventral and ventro-lateral border of the forth ventricle in the dorsal pons. Functionally and anatomically, the Edinger-Westphal nucleus, the nucleus of Darkschewitsch and the interstitial nucleus of Cajal (oculomotor and trochlear nuclei) and the dorsal raphe nucleus are not integrated into the PAG although they provide a major part of the gray matter ventral to the midbrain aqueduct (Bandler et al., 1991). The midbrain tegmentum laterally to the PAG is usually considered to be separated from the PAG by tectobulbar and tecto-spinal fibers and the fibers of the mesencephalic trigeminal tract. In the literature some disagreement can be found on how to best subdivide the PAG. The organizational classification of the periaqueductal gray shown in FIG. 4 follows the suggestion of Bandler and coworkers and is strictly based on anatomical and functional specificity expressed in longitudinal neuronal columns along the rostro-caudal axis of the PAG (Bandler et al., 1991). This work strictly follows this classification.

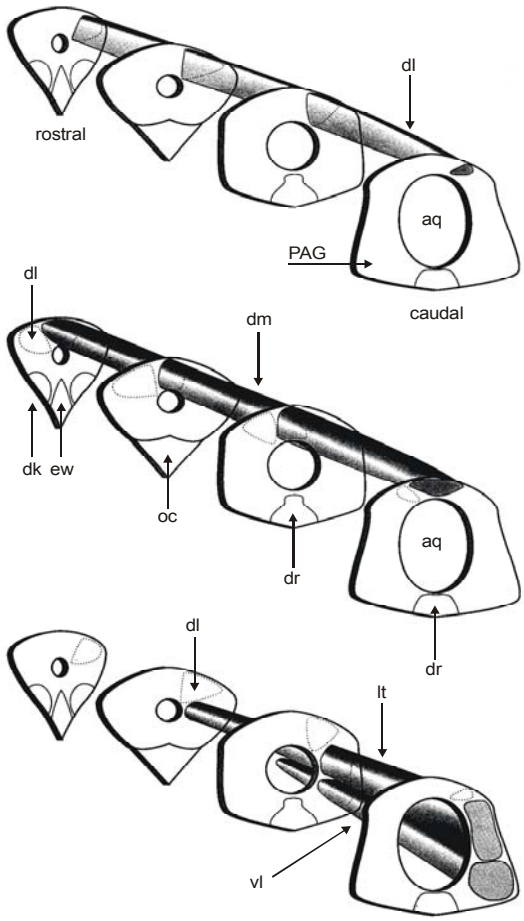

FIG. 4: The longitudinal columnar organization of the periaqueductal gray.
Top drawing) The dorso-lateral column is significant throughout the rostral and intermediate periaqueductal gray. A large number of GABA immunoreactive cells and higher densities of kainate binding sites than in other parts of the rostral and intermediate periaqueductal gray can be found here. Very little is known about the function of this column. *Middle drawing*) The dorso-medial column is represented throughout the entire rostro-caudal extend. Other than the dorso-lateral column heavy projections to the caudal brainstem can be found, although not much is known about its functional significance. *Bottom drawing*) Lateral and ventro-lateral column. The lateral column extends throughout the rostral and intermediate thirds of the region. Autonomic and somatomotor activities together with specific forms of defense behavior are associated with this region. Functional studies support the existence of a ventro-lateral column. Injections of excitatory amino acid agonists in this region evoke effects opposite of those evoked from the lateral column (decreased autonomic and somatosensory activity). *Abbreviations*: aq, aqueduct; dk, nucleus of Darkschewitsch; dl, dorso-lateral column; dm, dorso-medial column; dr, dorsal raphe nucleus;

ew, nucleus of Edinger Westphal; lt, lateral colum; oc, oculomotor nucleus; PAG, periaqueductal gray; vl, ventro-lateral column. Adapted, from Bandler (1991).

Additional support against the assumption that the periaqueductal gray serves as a center for vocal pattern coordination comes from earlier experiments by Jürgens and Pratt (1979) in which they showed, that electrical or pharmacological stimulation of the periaqueductal gray yields natural vocalizations, whereas stimulation of the reticular formation yields abnormal vocalizations. The normal acoustic structure of periaqueductally elicited calls (in contrast to artificial character of reticular elicited calls) was interpreted as the result of an indirect activation of the vocal pattern coordination mechanism. The periaqueductal gray probably acts more as a vocal gating mechanism rather than a vocal pattern generator (Düsterhoft er al., 2000).

2.3.3 The final common pathway for vocalization

Jürgens describes a hierarchically organized vocal pathway including the projection from the periaqueductal gray towards the neural network consisting of the parvocellular and dorsal medullary reticular formation, nucleus retroambiguus and solitary tract nucleus (Jürgens, 2002), for details see chapter 2.3.1 and 2.3.2. In his opinion the periaqueductal gray exerts a gating function in controlling the release of vocal patterns.
Holstege proposed that vocal pattern generation takes places within a final common pathway for vocalization originating from the periaqueductal gray and projecting to the nucleus retroambiguus (Holstege, 1989). This is an alternative view of the proposal that the PAG regulation of vocalization is achieved somewhat diffusely across the parvocellular reticular formation of the lower brainstem (Jürgens & Pratt, 1979; Thoms & Jürgens, 1987). In his anatomical study, Holstege found bilateral projections from the lateral part of the caudal periaqueductal gray to the nucleus retroambiguus. Since this nucleus projects to motoneuronal cell groups innervating mouth-opening and perioral muscles, intercostals and abdominal muscles as well as to motoneurons innervating the pharynx, soft palate and tongue (all these muscles are active in vocalization), he

concluded that the projection from the periaqueductal gray controls via the nucleus retroambiguus and this final common pathway the pattern generation for vocalization.

Vanderhorst and coworkers clearly traced projections from the periaqueductal gray to the nucleus retroambiguus in rhesus monkeys supporting the results on a periaqueductal-retroambiguus pathway. Their results showed that a compact group of neurons in the medial part of the lateral periaqueductal gray sends a dense and direct projection to the nucleus retroambiguus (Vanderhorst er al., 2000).

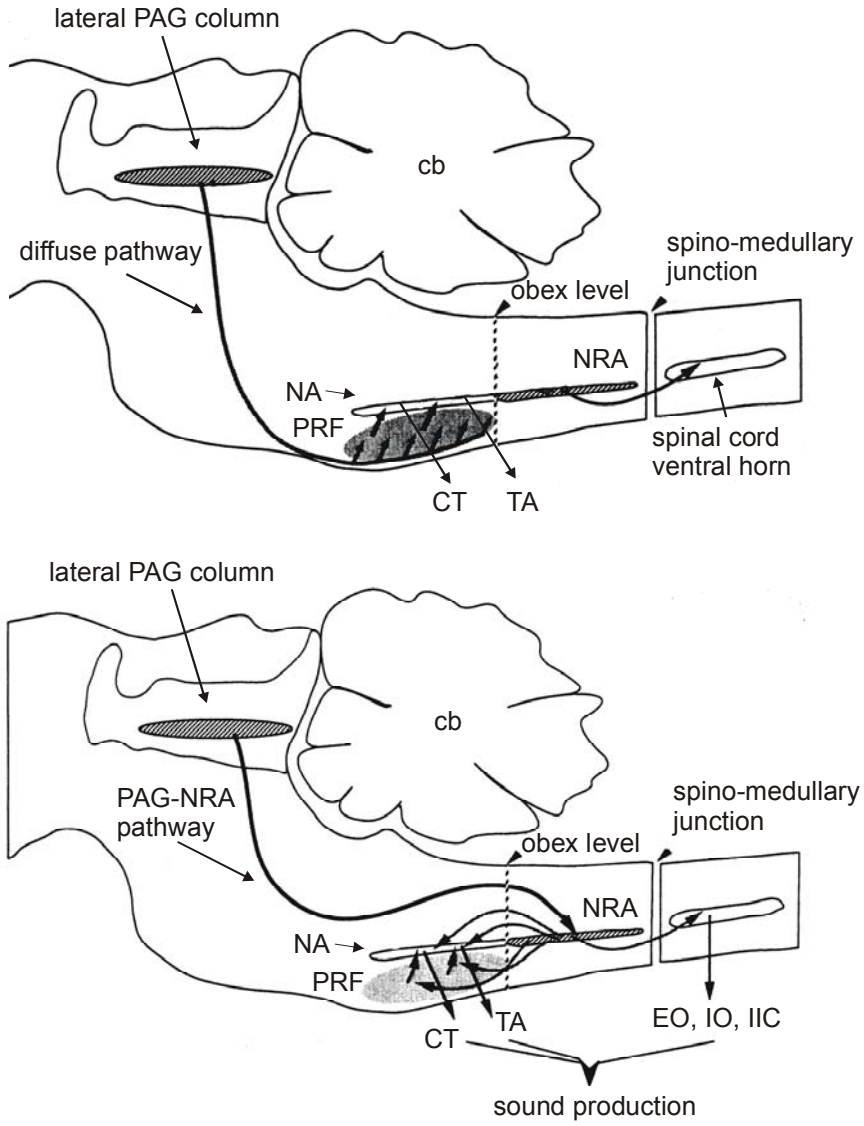

FIG. 5: The final common pathway for vocalization.
Possible representations of output projections from the periaqueductal gray. Adapted, from Zhang (1995). *Top drawing*) Projection from the periaqueductal gray towards reticular interneurons in the vicinity of

laryngeal motoneurons in the caudal medulla around the nucleus ambiguus (diffuse pathway) after Jürgens (Jürgens & Pratt, 1979; Thoms & Jürgens, 1987). *Bottom drawing*) The final common pathway as reported by Zhang and co-workers (1995). The periaqueductal gray projects directly to the nucleus retroambiguus, which itself controls the larynx muscles and expiratory muscles. *Abbreviations*: cb, cerebellum; NA, nucleus ambiguus; NRA, nucleus retroambiguus; PAG, periaqueductal gray; PRF, formatio reticularis. Muscles: TA, thyroarytenoid muscle; CT, cricothyroid muscle; EO, external oblique muscle; IIC, internal intercostals, IO, internal oblique muscle.

As shown in FIG. 5, the nucleus retroambiguus projects to the nucleus ambiguus, where motoneurons innervating structures such as larynx, pharynx and soft palate are located. The discussion about a final common pathway is complicated by the fact, that several authors found direct projections from the periaqueductal gray to the nucleus ambiguus: Vanderhorst and coworkers (2000) described projections from the periaqueductal gray to the nucleus ambiguus being much sparser than projections from the periaqueductal gray to the nucleus retroambiguus or from the nucleus retroambiguus towards the nucleus ambiguus and stress the importance of the periaqueductal-retroambiguus-ambiguus pathway being important for vocalization. They conclude that the ambiguus projections could serve the control of laryngeal vocal fold, orofacial and swallowing movements or it could play a role in respiration.

In an anatomical study using retrograde and anterograde tracing techniques Ennis and coworkers found neurons projecting from three distinct subregions of the periaqueductal gray to the nucleus ambiguus (Ennis et al., 1997). Interestingly the authors found that inputs from the medial preoptic area (a region associated with regulation of maternal and sexual behavior) terminating in the periaqueductal gray[1] at sites, heavily target the nucleus ambiguus. This projections could represent a circuit for the control of vocalization and cardiovascular adjustments during reproductive behavior (Ennis et al., 1997), since a part of the nucleus ambiguus controls parasympathetic neurons that innervate the heart (Bieger & Hopkins, 1987). Linking periaqueductal projections to the

[1] It must be emphasized that the periaqueductal gray is not only involved in control of vocalization. Somatic and autonomic responses as well as complex behavioral responses such as freezing behavior, flight, defense behavior, respiration, lordosis, cardiovascular adjustments and nociceptive inhibition are under the influence of the periaqueductal gray and can be elicited or manipulated with artificial stimuli, e.g. electrical microstimulation.

nucleus ambiguus with the control of vocalization only on the base of neuroanatomical results, as done by Dennis and coworkers (see above) does not seem to be very expressive. Neuroanatomical studies must be combined with electrical microstimulation experiments and selective lesion experiments to establish a functional involvement of particular areas in the control of vocalization.

3. Role of the periaqueductal gray in vocalization – a brief summary

Although the contribution of the periaqueductal gray to the control of different behaviors is not clear in detail, there is current agreement on its crucial role in control of vocalization:

- Vocalizations can be elicited in the periaqueductal gray in numerous animals and man: in the rat (Yajima et al., 1980), guinea pig (Martin, 1976), bat (Schuller & Radtke-Schuller, 1990; Suga et al., 1973; Valentine et al., 2002), cat (Hunsberger, 1956), squirrel monkey (Jürgens & Ploog, 1970), rhesus monkey (Magoun et al., 1937), gibbon (Apfelbach, 1972), chimpanzee (Brown, 1915) and man (Sem-Jacobsen & Torkildsen, 1960).

- vocalizations elicited in the periaqueductal gray generally resemble natural calls (Jürgens, 1994; Vanderhorst et al., 2000).

- The obvious lack of correlation between vocalization and emotional reaction indicates that periaqueductally evoked vocalizations do not represent secondary reactions to stimulation-induced motivational changes but are triggered more directly (Jürgens, 1994; Jürgens, 2002).

- The periaqueductal gray displays very short latencies for vocalization when being stimulated with electrical microstimulation. In the squirrel monkey latencies down to 50 ms where found within the periaqueductal gray (Jürgens, 2002).

- While in most other vocalization-eliciting areas repetitive stimulation causes a vanishing of the vocal reaction, periaqueductally evoked vocalization lasts for the whole duration of the stimulation showing no habituation to the stimulus (Jürgens, 1994; Jürgens, 2002).

- Lesions invading the periaqueductal gray can cause mutism without general akinesia. Lesions do not have to destroy the periaqueductal gray completely, as partial lesion leads to a loss of some vocal reactions, leaving others intact (Jürgens, 1994).

- Single-unit recording studies in the periaqueductal gray showed vocalization-correlated activity changes in several animals: in the cat (Adams, 1968), bat (Suga & Yajima, 1988), squirrel monkey (Düsterhoft et al., 2000) and macaque spec. (Larson & Kistler, 1984). A systematic relationship between periaqueductal activity changes and fundamental frequency changes of calls has not been demonstrated so far (Düsterhoft et al., 2000). Most of the cells found in this study did not even differentiate between individual call types.

- Afferent projections of relevance for vocal behavior reach the periaqueductal gray from essentially two groups of structures (Jürgens, 1994): The first group, when electrically stimulated, leads to the production of vocalizations. These structures all belong to the limbic system (see FIG. 2 fore details) depending upon an intact PAG for their production of vocalization (Fernandez de Molina & Hunsperger, 1962; Jürgens, 1982; Jürgens & Pratt, 1970; Jürgens & Pratt, 1979). The second group consists of sensory relay structures. They provide the periaqueductal gray with visual, auditory, gustatory and somatosensory information (Jürgens, 1994). Massive efferent projections where found from the periaqueductal gray to the reticular formation which itself is connected with all phonatory motor nuclei

(Holstege, 1989; Jürgens & Pratt, 1970; Thoms & Jürgens, 1987). Other efferents from the periaqueductal gray reach the nucleus retroambiguus, a group of premotor neurons (Vanderhorst et al., 2000) and the nucleus ambiguus.

4. Vocalization in bats

Bats use vocalizations for communication and for echolocation. Like in any other mammal, social calls are used for intraspecific interactions. Echolocation in general is used for orientation gathering information about the environment, despite the possible situation that echolocation calls of one bat could serve another bat in a non-intentional *communicative* manner to indicate potential food sources (Fenton, 1984). Echolocation capability was developed independently from some taxa among the following groups: shrews, birds, tenrecs, cetaceans, rodents, bats (Busnel & Fish, 1980) and seals (Renouf & Davis, 1982). While most of these echolocators use brief broadband clicks, which are unstructured and easy to produce, microchiroptera produce calls structured in time (Fenton, 1984).

Communication calls and echolocation calls can clearly be separated from each other acoustically by their specific spectral patterns. Although clearly distinguishable, under physiological aspects the production of echolocation calls must access the same laryngeal and expiratory components as the production of communication calls. The current discussion on control of vocalization together with the functional brain areas involved in it could take advantage from results found in bats.

4.1 Echolocation calls can be elicited in the anterior cingulate cortex

Since the beginning of systematic research on vocalization related sites within the vertebrata central nervous system in the late 1960s, a variety of different animals had been under investigation (see chapter 2 for an overview). Interestingly, bats which have a very specialized vocalization system were never in the center of attention. These animals utter echolocation calls and species-specific communication calls produced by the larynx while shaping by the vocal tract is very limited (in contrast to humans where articulation is the main shaping mechanism (Ploog, 1988)). Production of these different types of calls in bats presumably shares the same neural control centers as in higher mammals (Schuller & Radtke-Schuller, 1990).

After a few experiments using electrical stimulation to explore vocally active sites within the brain of bats were published (Suga et al., 1973; Suthers & Fattu, 1982), it was not until 1987 that a rather systematic work was done on the elicitity of vocalizations in the anterior cingulate cortex of bats (Gooler & O´Neill, 1987). The authors mention that echolocation calls, virtually indistinguishable from natural calls can be elicited by microstimulation within this region. Posterior to this region audible calls resembling social calls can also be elicited with microstimulation. From there findings they finally concluded that vocalization, if uttered in the purpose of social communication [this applies for bats as well as for higher primates like monkeys] or for foraging and navigation in echolocating bats, must be a highly motivated behavior (Gooler & O´Neill, 1987).

4.2 The periaqueductal gray in bats

For the periaqueductal gray it was shown by stimulation experiments that echolocation calls as well as calls of non-echolocation type (Suga et al., 1973; Valentine et al., 2002) could be elicited in bats. Suga and coworkers elicited sounds similar to orientation signals in the genus *Myotis* from the lateral part of the periaqueductal gray (Suga et al., 1973). In *Pteronotus parnellii* and *Pteronotus suapurensis*, stimulation of the periaqueductal gray yield vocalizations indistinguishable from natural orientation sounds and for *Eptesicus fuscus* and *Noctilio leporinus*, electrical stimulation elicited vocalizations similar to natural orientation sounds (Suga et al., 1973). The authors also reported that stimulation of the ventro-medial area of the periaqueductal gray in *Myotis* elicited vocalizations quite different from orientation sounds, but these vocalizations were in association with various large body movements. For *Eptesicus fuscus* it was shown by Valentine and coworkers (2002) that electrical stimulation of the periaqueductal gray leads to vocalizations comparable to communication signals emitted by this species.

But a systematic work on the elicitation of different types of communication calls on the one hand and echolocation calls on the other hand applying the same criteria on

triggered vocalizations as it was done for higher primates (short latencies of triggered calls, no habituation to stimuli, low thresholds) is not available to date.

4.3 Other neuronal substrates in bats yielding echolocation calls

On the search of brainstem areas where electrical microstimulation could trigger natural echolocation calls, three areas were found where vocalizations could be elicited without temporal or spectral distortions (Schuller & Radtke-Schuller, 1990): The deep layers of the superior colliculus, the deep mesencephalic nucleus in the reticular formation and a restricted area medial to the rostral parts of the dorsal nucleus of the lateral lemniscus. To ensure that the brain areas were “specific” for the production of biosonar vocalizations, the authors applied the following criteria:

a) Threshold currents for triggering vocalizations were below 10 µA.
b) Vocalizations corresponded to spontaneously produced echolocation calls with respect to spectral and temporal patterns.
c) No body movements other than of ear, mouth or nose accompanied vocalizations.
d) Latency between onset of vocalization and stimulus train was stable and below 100 ms.
e) Vocalizations were not uttered as a consequence of stimulus-induced arousal of the animal.

The shortest latencies (mean latency: 30.9 $\pm$ 9.1 ms s.d.) for eliciting echolocation calls at lowest thresholds ($<$ 10 µA) and/or pinna movements not accompanied by any arousal of the animal where found in the area medial to the dorsal nucleus of the lateral lemniscus (Schuller et al., 1997). Strikingly, by fulfilling the above listed requirements, this area shares most of the criteria which awarded the periaqueductal gray a crucial role in the production of communication calls.

4.4 The paralemniscal tegmental area

The expression “paralemniscal tegmental area” only expresses the position of the restricted area medial to the rostral parts of the dorsal nucleus of the lateral lemniscus and does not describe any identified nucleus. A discussion is complicated by the fact that different authors had different motivations to investigate this area, which led to different functional or anatomical findings suggesting rather an aggregation of different fields than a clearly delimited area:
On the basis of neural responses to acoustic stimuli (pure tones, frequency-modulated sweeps and noise bursts) Covey (1993) differentiated between a dorsal paralemniscal nuclei (DPL), located rostral to the dorsal nucleus of the lateral lemniscus and separated it from the paralemniscal zone (PL) and the dorsal nucleus of the lateral lemniscus (DNLL). At monaural presentation of the stimuli, most of the neurons in the DNLL and in the PL were activated by contralateral sound, and also in the DPL all tested neurons responded only to a contralateral sound. In a binaural testing situation (contralateral stimulus at 20 dB above threshold, ipsilateral sound level varied) the most common response pattern in all three areas was suppression of the contralateral response to ipsilateral sound level increase. In the DPL, 29% of all neurons tested showed binaural responses, in the DNLL 84% of all neurons showed binaural responses and in the PL 88% were binaural. Thus the author reserved an auditory function for the DPL.

Henkel and Edwards defined a tegmental area in the cat as the paralemniscal zone and investigated this area under the aspect of pinna movements (Henkel, 1981; Henkel & Edwards, 1978). In their anatomical studies using orthograde and retrograde tracers they injected HRP (horseradish peroxidase) into pinna muscles to determine the facial nucleus regions projecting into these muscles. Additionally brainstem regions projecting to the facial nucleus were identified with HRP and superior colliculus projections to these areas were identified autoradiographically. They concluded that superior colliculus control of pinna movements is mediated by the paralemniscal zone via indirect connections to the facial nucleus. HRP injections into the paralemniscal zone showed also afferents from the external nucleus of the inferior colliculus and the periolivary cell group (auditory afferents), the nucleus prepositus hypoglossus, the adjacent pontine

reticular formation and the vestibular nuclei (premotor regions, among other things involved in gaze control), the nucleus cuneiformis and the periaqueductal gray (Henkel, 1981). Since they found relatively sparse labeling in the auditory regions, Henkel concluded that “sensorimotor” integration necessary to guide pinna movements does not take place primarily in the lateral midbrain tegmentum and mentions that the inferior colliculus is as likely to be the site for this kind of “sensorimotor” integration.

In research related to oculomotor questions the tegmental region was termed lateral tegmental region (LTR), where neurons, probed in the cat responded to visual, vestibular and auditory stimulation (Gerlach et al., 1991; Gerlach & Their, 1995). Their anatomical studies using HRP and fluorescent tracers, injected into different sites of the LTR, revealed afferents from the vestibular nuclei, the nucleus prepositus hypoglossus, the superior colliculus, the periaqueductal gray and the contralateral LTR. According to the authors, the afferents from the superior colliculus could be the source of visual signals fed into the LTR while head movement-related information could come from the nucleus prepositus hypoglossus and the vestibular nuclei (Gerlach & Their, 1995).

In a neuroanatomical study using *Rhinolophus rouxi*, Metzner (1996) found evidence that the paralemniscal tegmentum (PL) could provide an interface between the pathways for auditory sensory processing and for the motor control of vocalization. WGA-HRP (wheat germ agglutinin conjugated to horseradish peroxidase), injected into different sites of the PL, yield afferents from the dorsal nucleus of the lateral lemniscus, the central nucleus and the rostral portion of the inferior colliculus, the lateral superior olive, the superior colliculus and the nucleus of the central acoustic tract (all are part of the auditory pathway). Efferents where found to the superior colliculus, the facial nucleus and the reticular formation rostral to the nucleus ambiguus. In an electrophysiological study the same author reported, that the activity of neurons in the PL was correlated to sound emission and auditory stimuli differentially. Responses also varied according to the time delay between an auditory stimulus and a preceding vocalization, while this delay-sensitivity completely disappeared when vocalizations were simulated acoustically. Since mainly vocalization and “echo-parameters”, which

occur in Doppler-shift compensation were encoded, Metzner (1989) emphasizes the role of the PL in this special behavior.

Schuller and coworkers (Schuller & Radtke-Schuller, 1990; Schuller et al., 1997) delimited the paralemniscal area in *Rhinolophus rouxi* using the ability to electrically elicit vocalizations (See chapter 4.3 for details). In a combined electrophysiological and neuroanatomical study, Schuller and coworkers found similar results for *Pteronotus p. parnellii* (Schuller et al., 1997). The paralemniscal tegmental area (PLA) was functionally identified as location, where vocalizations could be elicited at thresholds below 10 µA. The authors describe three different sizes for neurons within the PLA (medium elongated cells, small elongated cells and small peripheral cells) and found that stimulation in the PLA was effective for eliciting vocalizations in an area which contains the medium elongated cells. This area was restricted to a zone ventral to the inferior colliculus. Ventrally it was bordered by the superior cerebellar peduncle and laterally by the nuclei of the lateral lemniscus. Its rostral limit reached the substantia nigra (Schuller et al., 1997). Stimulations of this area elicited vocalizations indistinguishable from spontaneously uttered echolocation calls at very short latencies (25 – 30 ms) without any motor activity except pinna movements. By placing WGA-HRP into vocally active sites of the PLA they found as main afferent connection projections from the superior colliculus. Using this input, the tegmental area might have motor controlling functions for pinna movements via efferent projections to the facial nucleus (Schuller et al., 1997). Additionally, auditory and visual information could be fed into the paralemniscal tegmental area after being processed across the superior colliculus (Schuller et al., 1997) and vestibular informations could come from afferent projections originating from the nucleus prepositus hypoglossus. Input also came from the substantia nigra [functional part of the basal ganglia, motor intention], the putamen [part of the striatum, a nucleus of the basal ganglia, modulation of motor impulses], the reticular formation [numerous functions such as control of respiration, circulation, vomiting, extrapyramidal motor pathway] and the contralateral paralemniscal tegmental area (Schuller et al., 1997).

On the efferent side projections of the tegmental area could be traced towards the putamen, the nucleus accumbens [part of the striatum, supposed to represent an

interface for the converting of motivation into action] the substantia nigra, the pretectal area [senso-motoric integration, (Nixdorf, 2003)] and the superior colliculus (Schuller et al., 1997). Efferent projections from the tegmental area also reached the cuneiforme nucleus, the parabrachial region and the periaqueductal gray (Schuller et al., 1997). The authors concluded that the PLA has a potential sensory-motor function: On the sensory side the PLA receives information coming from superior colliculus (visual and auditory) and from the nucleus prepositus hypoglossus (vestibular). Motor involvement in vocalization and pinna movements could be realized via the nucleus facialis and the nucleus cuneiformis. The PLA does not interfere with low level motor control, but rather might play a role in co-ordinating emission of echolocation calls or start of pinna movements with other behaviors such as head orientation (Schuller et al., 1997).

4.5 The paralemniscal tegmental area – a brief summary

The paralemniscal tegmental area has been considered from different perspectives. Several different functional involvements have been characterized, but it seems hard to compare the different results in terms of a uniform paralemniscal tegmental area:
The “paralemniscal zone” of the cat defined by Henkel and Edwards (see above) corresponds partly to the connectional patterns found in bats, but the area defined with electrical microstimulation in the bat is more restricted and only occupies the dorsal half of the paralemniscal zone as defined by Henkel and Edwards (Schuller et al., 1997).
Discrepancies in connectional patterns between findings from Metzner (see above) and Schuller (see above) and thus different interpretations of functional involvements of the paralemniscal tegmental area can most probably be attributed to differences in the location of tracer injection sites (Schuller et al., 1997).

In general, the definitions of the paralemniscal area and surrounding regions are different in different species of bats or in different mammals so that direct comparisons of the tegmental area have to be treated with caution.

5. Central questions of this thesis

This thesis strictly follows the definition suggested by Schuller and coworkers on a sensory-motor function of the paralemniscal tegmental area.

A direct vocal controlling pathway from the periaqueductal gray towards the nucleus retroambiguus (as outlined by Zhang, see FIG. 5) or from the periaqueductal gray to the reticular formation (as outlined by Jürgens, see FIG. 5) seems to be insufficient to explain findings from Schuller and coworkers on the involvement of the paralemniscal tegmental area in motor control of vocalizations.

This leads to the following questions:

- How does the paralemniscal area interfere with the final common pathway for vocalization in bats?

- How does the paralemniscal area interfere with the production of echolocation calls and communication calls in bats?

- How does the paralemniscal area and the periaqueductal gray interfere with each other in terms of the production of echolocation calls and communication calls?

- How does the periaqueductal gray interfere with the final common pathway for vocalization in bats?

- How does the periaqueductal gray interfere with the production of echolocation calls and communication calls in bats?

IV. Aims and achievements of the Thesis

The main intention of this Ph.D. project was to understand the involvement of the periaqueductal gray and the paralemniscal area in the production of communication calls and echolocation calls in the animal model *Phyllostomus discolor*. It is the first time that the production mechanisms of these different types of vocalizations are investigated in the same animal. To gain insight into mechanisms of neural control of vocal behavior at the level of these two brain regions several approaches were used:

(1) With its rich repertoire of about 20 different social calls (Esser & Pistohl, 1998) and at the same time its usage of stereotyped frequency modulated echolocation calls (Rother & Schmidt, 1982), the bat *Phyllostomus discolor* represents an ideal animal model for investigations on control of both types of vocalizations. Together with Andreas Nixdorf (Ph.D. student) a preliminary stereotaxic brain atlas of this species (unpublished) was developed in order to have stereotaxic access to brain regions.
The results are described and discussed in detail in paper 1, a brief discussion is given in chapter V, part 1.

(2) Identification of the periaqueductal gray and the paralemniscal area in *Phyllostomus discolor* was carried out electrophysiologically and neuroanatomically. With electrical microstimulation several types of communication calls and echolocation calls could be elicited within the periaqueductal gray and echolocation calls in the paralemniscal area. Delimited areas of the two brain regions could be linked to individual types of calls for the first time in this species. In histologically processed brain sections the two brain areas could be identified and delimited towards surrounding areas.
Results are described and discussed in detail in paper 1, a brief discussion is given in chapter V, part 1.

(3) In order to exclude activation of fibers of passage within vocally active areas of the periaqueductal gray and the paralemniscal area as source of vocal responses, kainic acid (GLU agonist) was locally applied using microdialysis. Communication calls could

be elicited in the periaqueductal gray, and echolocation calls could be triggered in the paralemniscal area upon pharmacological stimulation, which demonstrated that stimulation of neurons and not fibers of passage are responsible for the vocal responses. Respiration is closely linked to vocalization and influenced by the stimulation of the two areas.

Results are described and discussed in detail in paper 1, a brief discussion is given in chapter V, part 2.

These results indicated that descending control for communication calls and echolocation calls seem to differ in its functional organization. The periaqueductal gray and the paralemniscal area may interact differentially with the final common pathway controlling vocalization.

(4) In order to test for the possibility of differentiated neural pathways for vocal control, electrodes for chronic electrical microstimulation were developed and implanted stereotaxically within the periaqueductal gray at sites, where echolocation calls or communication calls could be elicited. Simultaneously, the paralemniscal area was reversibly blocked by kynurenic acid (GLU antagonist). The results showed that periaqueductally induced echolocation calls depend on an intact paralemniscal area contralateral to the stimulation site. The emission of communication calls upon electrical stimulation within the periaqueductal gray cannot be influenced by such blockades of the paralemniscal area.

Results are described and discussed in detail in paper 2, a brief discussion is given in chapter V, part 3.

(5) Tracer studies with WGA-HRP in vocally active sites of the periaqueductal gray showed projections towards the region of the nucleus ambiguus/retroambiguus complex without well defined terminal endings in this area. The nucleus ambiguus was identified by its cholinergic properties using the AChE staining procedure.

Results are described and discussed in detail in paper 2, a brief discussion is given in chapter V, part 4.

The results can be summarized as follows: A vocal pathway leading from the periaqueductal gray directly to the nucleus ambiguus cannot explain the involvement of the paralemniscal area on the production of echolocation calls. Although direct connections between the periaqueductal gray and the paralemniscal area could not be demonstrated, the physiological experiments suggest a functional connection of these two brain areas, at least for the production of echolocation calls.

It can be concluded that the paralemniscal area is essential for the production of periaqueductally induced echolocation calls but not for particular types of communication calls. The control of particular types of communication calls on the one hand and echolocation calls on the other hand may be realized via different vocal pathways being more complex than the direct periaqueductal-nucleus retroambiguus pathway suggested in the literature.

The periaqueductal gray and the paralemniscal area do interact differentially with the final common pathway for vocalization.

V. Discussion

(1) The relative location of the periaqueductal gray in *Phyllostomus discolor* is similar to what is found in other mammals like rats (Van Bockstaele et al., 1991) or other bats (Suga et al., 1973). The region of the paralemniscal area in *Phyllostomus discolor* is similar to what is found in other bats such as *Rhinolophus rouxi* (Metzner, 1993; Radtke-Schuller, Anat. Anst. LMU, Munich, Germany, brain atlas, unpublished) and *Myotis montivagus* (Baron et al., 1996).

The periaqueductally induced vocalizations in *Phyllostomus discolor* can be subdivided into echolocation calls and several types of communication calls. In behavioral experiments it has been shown that this bat species has a repertoire of at least 20 different social calls (Esser & Pistohl, 1998). The stimulation experiments carried out in this thesis did not yield all communication calls described by Esser and Pistohl. The majority of periaqueductally induced social calls resemble a group of calls which was described as aggressive calls by Esser and Pistohl. This dominance of aggressive calls obtained in an experimental situation is confirmed by findings in cat and monkey, where stimulation experiments also induce call types of agonistic kind more often than calls related to other behaviors (Jürgens, 1994; Jürgens, 2002). The constraints of the experiment could provoke an agonistic disposition of the animal encouraging vocalizations of agonistic kind.

Periaqueductally induced echolocation calls of *Phyllostomus discolor* closely resemble natural echolocation calls in their spectral composition, whereas the duration of the calls shows a wider variability than described for spontaneous calls emitted in laboratory (Rother & Schmidt, 1982). The natural variation of echolocation calls of free moving bats in the field is generally larger than recorded in the laboratory (Surlykke & Moss, 2000), reflected in longer durations, longer interpulse intervals and a greater variability in bandwidth.

Electrical stimulation in the paralemniscal area elicits exclusively echolocation calls showing the typical harmonic structure of *Phyllostomid* echolocation calls (Esser & Daucher, 1996; Rother & Schmidt, 1982) with small variations. Since these authors

recorded calls from freely moving bats in the laboratory the differences in duration and bandwidth may again not represent the real range of these parameters.

Vocalization-eliciting sites in the periaqueductal gray overlap to a large extend and are not organized in distinct clusters for individual call types (Jürgens, 1994). Such overlap can also be found in *Phyllostomus discolor* and stimulation at identical sites can result in the emission of different call types in subsequent penetrations. Therefore different types of calls are possibly elements of the same behavior and stimulus locations might rather represent a behavioral context than a particular type of vocalization.

Short latencies and low thresholds together with the lack of habituation to repetitive stimuli of periaqueductally and paralemniscally induced vocalizations support the assumption that both areas have a relatively direct access to the descending vocal pathway system.

(2) Kainic acid was effective in both the periaqueductal gray and the paralemniscal area. This Glutamate agonist (selective at kainite receptors) activates specifically neurons and not fibers of passage originating from remote brain areas.

Delayed onset of vocalization to microdialysed kainic acid may be explained with slow diffusion dynamics of the agonist. The delayed fading and offset of vocalization after completion of microdialysis can be explained with slow dilution of the substance within brain tissue.

Application of kainic acid in the periaqueductal gray provokes an increase of respiration rate starting always earlier than the onset of kainic acid-induced vocalizations. The control of respiration rate and the triggering of vocalizations seem to depend on separate mechanisms. An adaptation of the respiration rate could also be linked to particular behaviors, e.g. different stages of defense (Fanselow, 1991) or hypotension plus decreased vasoconstrictor tone to allow the animal to rest and recuperate efficiently (Carrive, 1991) are modulated by periaqueductal gray stimulations. The periaqueductal gray could therefore serve as an integrator between a particular behavior and the adequate respiration rate.

The same pattern of delayed vocalization onset after respiration rate increase is found in the paralemniscal area. Modulation of respiratory rate by the paralemniscal area may either use a separate pathway or be a result of coactivation of periaqueductal circuits.

(3) Are different classes of vocalizations (communication vs. echolocation calls) processed by separate vocal pathways in the bat? The paralemniscal area and the periaqueductal gray are involved in vocal control, with the distinction that communication calls cannot be elicited in the paralemniscal area, whereas echolocation calls can be triggered in both regions.

The experiments demonstrated a functional linkage between both areas for the production of vocalizations, at least what concerns the control of echolocation calls. Echolocation call, but not social call emission triggered in the periaqueductal gray was dependent on an intact contralateral paralemniscal area, which shows that there are functional differentiations between control mechanisms for communication call and echolocation call production.

Communication calls would be controlled by a pathway described by several authors (Ennis et al., 1997; Zhang et al., 1994; Zhang et al., 1995). In this pathway a direct connection from the periaqueductal gray towards the nucleus retroambiguus is proposed. This nucleus includes premotor neurons which send projections to thoracic and upper lumbar motoneurons (Vanderhorst et al., 2000; Vanderhorst et al., 2001) involved in expiration, and to the nucleus ambiguus containing laryngeal and pharyngeal motoneurons (Holstege, 1989; Vanderhorst et al., 2000; Vanderhorst et al., 2001). This network is sufficient to explain the unimpaired production of communication calls but cannot explain the findings that periaqueductally induced echolocation calls can be blocked by paralemniscal inactivation. A possible “echolocation call pathway” originating in the periaqueductal gray must therefore integrate paralemniscal activity at some stage, which has not yet been determined.

(4) Several authors report a direct projection from the periaqueductal gray to the nucleus retroambiguus (Gerrits & Holstege, 1996; Zhang et al., 1995), a nucleus displaying functional connections with the rostrally adjacent nucleus ambiguus. Dense projections from the periaqueductal gray towards the region of the nucleus ambiguus were found for *Phyllostomus discolor,* although discrete terminal endings were not identified. It is unclear whether a nucleus retroambiguus can be distinguished anatomically for this species. Projections from the periaqueductal gray aim to this caudal region which could correspond to the nucleus retroambiguus as defined in other mammals.

The preliminary data point to a direct periaqueductal / nucleus retroambiguus pathway in *Phyllostomus discolor* as implementation of vocal control, for at least a subgroup of communication calls. Other, more indirect pathways for neural control of vocalization, at least for the control of echolocation calls, must exist as the experiment with paralemniscal blockade suggest.

However, no direct projections from the periaqueductal gray to the paralemniscal area, as described by other authors (Gerlach & Their, 1995; Schuller et al., 1997) could be traced in *Phyllostomus discolor* so far. The reason may be that the projections of the periaqueductal gray to the paralemniscal area are of different density in different species and could therefore have escaped from detection in *Phyllostomus discolor*.

On the other hand, involvement of the paralemniscal area would not necessitate a serial "periaqueductal – paralemniscal connection" but could influence vocal production in a parallel way to a common vocal pathway.

VI. Summary and conclusion

Echolocation calls and communication calls can be elicited with electrical microstimulation in the periaqueductal gray. The periaqueductal gray, involved in the neural control of communication calls as well as echolocation calls, constitutes part of the vocal pathway. The periaqueductal gray would contribute an integrator for particular behaviors and its appropriate vocalizations under both conditions (echolocate and communicate). The paralemniscal area, in terms of vocalization exclusively controls echolocation calls. Reversible blockade of the vocally active paralemniscal area totally blocks periaqueductally induced echolocation calls leaving communication calls triggered in the same region unaffected. The paralemniscal area therefore seems to be essential for periaqueductally induced echolocation calls but not for particular types of communication calls. This suggests a differentiated organization of vocal pathways for certain types of communication calls and echolocation calls, and suggests that the descending vocalization system is more complex than the network connecting the periaqueductal gray directly to the nucleus ambiguus/retroambiguus complex.

VII. Things to do ...

We are far from understanding neural control of vocalization in every detail. To help putting together the pieces of the puzzle research on vocalization systems in bats could contribute through several approaches:

Tracer experiments using anterograd and retrograde tracers injected into vocal active sites of the periaqueductal gray and the paralemniscal area should bring more insight into vocally relevant interconnections. Since different types of communication calls and echolocation calls can be triggered at different sites within the periaqueductal gray, precise applications of tracers into the individual vocally active sites could reflect differentiated vocal pathways.

A reversible blockade of the paralemniscal area has no influence on the production of a particular type of communication call. It would be of great interest to know how such blockades would affect other types of communication calls. The experiments with blockades of the paralemniscal area therefore should be continued testing all types of communication calls potentially triggered in the periaqueductal gray on its ability to survive such blockades. These findings could uncover whether differentiated vocal pathways discriminate only between echolocation calls and communication calls or whether more global aspects of vocalizations, like call structure etc., constitute criteria for this distinction.

Single cell recordings in the paralemniscal area with simultaneous elicitation of vocalizations in the periaqueductal gray could give insight into a possible interplay of both areas. These recordings should also be executed in the periaqueductal gray during electrical stimulation of the paralemniscal area. Additionally simultaneous electrical stimulations of both structures could reveal access priorities of one or the other structure on the control of vocalization. This set of experiments could be completed with independent electrical stimulations in both areas together with single cell and multi unit recordings in the nucleus ambiguus/retroambiguus complex.

The findings that respiration can be influenced by stimulations in the periaqueductal gray and paralemniscal area is another aspect of interest. Stimulation experiments in both areas together with single cell or multi unit recordings in the pre-Bötzinger complex for example, an area within the groups of respiratory neurons containing all classes of neurons important for respiratory rhythm generation (Connelly et al., 1992; Schwarzacher, 1995) should shed light on the functional interaction of respiratory and vocal control.

On a long scale experiments with *Phyllostomus discolor* with its very stereotypic echolocation calls and a variety of communication calls could help on the search of the vocal pattern generator, one, in my opinion central questions of research on vocalization systems.

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Paper 1

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Periaqueductal gray and the region of the paralemniscal area have different functions in the control of vocalization in the neotropical bat, *Phyllostomus discolor*

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Keywords: communication, echolocation, kainic acid, PAG, PLA

Abstract

The periaqueductal gray matter and the region of the paralemniscal area were neuroanatomically delineated in the brain of the neotropical bat *Phyllostomus discolor* [Wagner (1843) *Arch. Naturgesch.*, **9**, 365–368] and were probed with electrical microstimulation for eliciting vocalizations. In a well-delimited rostral portion of the periaqueductal gray exclusively, communication calls could be triggered at low stimulation currents. Communication calls as well as echolocation calls could be elicited at the dorsal and ventral edges of this area. Pharmacological stimulation with microdialysed kainic acid in this particular periaqueductal gray area demonstrated that neurons and not fibres of passage are activated for triggering vocalization. Solely echolocation calls were emitted upon electrical microstimulation or with microdialysed kainic acid in the region of the paralemniscal area. The periaqueductal gray appears to be involved in vocal pathways that control both communication calls and echolocation calls, while the region of the paralemniscal area seems to be specialized for control of echolocation calls only. Respiration is similarly influenced by stimulation in the periaqueductal gray and the region of the paralemniscal area. Periaqueductal gray and paralemniscal area interact differently with the final common pathway for vocalization, and may represent different functional organization in the vocal controlling pathways for communication calls and echolocation calls.

Introduction

The control of echolocation calls in bats has been dealt with in numerous publications (Suga *et al*., 1973; Metzner, 1996), whereas communication call control has been neglected in these mammals so far, although bats have large repertoires of communication calls (Esser, 1990; Esser, 1994; Esser & Daucher, 1996). In general, echolocation calls and social calls can clearly be distinguished acoustically from each other by their spectral pattern and different kinds of vocalizations refer to clearly distinct behavioural contexts (i.e. communicating and orientating). In this context the question arises whether the vocal control also shows differences in the neural and functional organization of vocal pathways. Social calls can be elicited by stimulation of the periaqueductal gray matter (PAG) as shown in a variety of mammals, including squirrel monkey (Jürgens, 1979; Lu & Jürgens, 1993), rhesus monkey (Larson & Kistler, 1984), rat (Yajima *et al*., 1980), guinea pig (Martin, 1976) and gibbon (Apfelbach, 1972). Additionally stimulating the PAG in bats evokes echolocation calls (Suga *et al*., 1973; Suga & Yajima, 1988) as well as calls of nonecholocation type (Suga *et al*., 1973; Valentine *et al*., 2002). This study outlines a more thorough examination of the types of social calls elicited by stimulation of the PAG in *Phyllostomus discolor*, a bat which has a rich repertoire of social calls.

The paralemniscal tegmental area (PLA), located in the dorsal tegmentum rostral and medial to the dorsal nucleus of the lateral lemniscus, is an integral part of audio-motor control of ultrasonic vocalization and pinna movements in *Pteronotus p. parnellii* (Schuller *et al*., 1997). In *Rhinolophus rouxi*, electrical microstimulation within that area elicits ultrasonic vocalization at lower stimulation currents and shorter latencies in comparison to all other vocal stimulation sites in the brainstem (Schuller & Radtke-Schuller, 1990). Both bat species, although belonging to different families, show striking parallels in echolocation call patterns (constant frequency–frequency-modulated, CF–FM) and in audio-vocal behaviour.

The paralemniscal tegmentum has been described in detail as an audio-vocal interfacing structure in *Rhinolophus rouxi*, and contains neurons responsive to auditory stimuli as well as neurons in its rostral part which are active during spontaneous vocalization (Metzner, 1993; Metzner, 1996). However, the paralemniscal tegmentum as described by Metzner extends more caudally and ventromedially than the paralemniscal tegmental area in which vocalization can be elicited as described by Schuller & Radtke-Schuller (1990).

In contrast to CF–FM bats, *Phyllostomid* bats typically emit brief multiharmonic echolocation calls which are characterized by a steep downward-directed frequency modulation (Esser & Daucher, 1996) at a faint sound pressure level (86 dB max), at least in stationary animals (Rother & Schmidt, 1982). This study examines the PLA of *Phyllostomus discolor* as a part of the vocal pathway responsible for the production of echolocation calls.

In the *Phyllostomid* bat the substrates for vocal control of echolocation calls (a region of the PLA) and of communication calls (the PAG) are investigated. The findings that communication calls and echolocation calls can be elicited by stimulation of the PAG while stimulation of the PLA only triggers echolocation calls demonstrates a possible functional and connectional difference in respect to vocalization of these two mesencephalic regions.

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Differentiated results concerning the organization of vocal control would foster a concept of partially separate pathways for echolocation calls and communication calls.

Materials and methods

Seven male neotropical bats, *Phyllostomus discolor*, were used for this study. All animals came from a pure male colony, separated from a mixed breeding colony. During the experiments, the particular animal under investigation was kept separate from the male colony but under the same seminatural conditions (12-h light: 12-h dark cycle, 65–70% humidity, 25–30 °C).

Surgical preparation of the animals was performed under Halothane® (Hoechst, Germany) anaesthesia with an initial dose of 3% Halothane® and additional 20–30-s doses of 1.5% Halothane® when needed to maintain the anaesthetic state of the animal. Skin and muscles covering the skull were in addition locally anaesthetized with 2% Xylocain® (Astia, Germany).

The skin and muscles overlying the skull were cut rostro-caudally along the midline, reflected to the sides and kept in place using dental sponge (Gelastypt®, Hoechst, Germany). Minor bleedings were staunched with the coagulant Orbat® (lege artis Pharma, Germany).

A small holding tube to allow later fixation of the head in a stereotaxic device during experiments was attached to the skull surface with a light-curing dental compound (Microglass®, Kulzer, Germany).

Throughout the experiment the wound margins were treated with Orbat® if needed and additionally 2% H_2O_2 and/or Betaisodona® (Mundipharma, Germany) were used to disinfect the wound margins. Later, during experiments the animals were awake.

The experiments were conducted in an acoustic chamber lined with structured foam to reduce acoustical interference from the environment and to minimize the reflections of call signals. The animals were placed in a sandwich-like holder which prevented gross body movements and the head was immobilized by attaching the surgically affixed tube to a head-holder that allowed accurate repositioning ($\leqslant 10$ μm) of the animal in the stereotaxic device throughout experimental procedures.

The orientation of the skull and consequently the brain within the stereotaxic coordinates was determined by scanning the profile lines of the exposed skull in parasagittal and transverse directions at the first postoperative day. Details of the stereotaxic device, the procedures to determine the skull position, and the reconstruction of the stimulation and microdialysis sites are described elsewhere (Schuller *et al*., 1986). This method typically yields accuracy better than 100 μm in all three dimensions. As a control of the stereotaxic procedure, the stimulation sites within the brain were marked by placing electrical lesions for post-experimental anatomical verification and comparison with the stereotaxically known coordinates. The lesions were made by delivering –2 μA DC for 5 min through the tip of the stimulation electrodes. Starting from the stereotaxically placed lesion with its known coordinates all stimulation sites could be calculated and compared with the three-dimensional coordinates of the brain area under investigation to verify the range of stimulation.

The stimulation experiments typically started on the third postoperative day and did not last longer than 5 h per day. To insert the small stimulation electrodes, a small hole was drilled into the skull using a miniature drill (homemade) while wound surfaces were treated with the surface active local anaesthetic, legecain (lege artis Pharma, Germany). Holes had a typical diameter of 200 μm and several probe penetrations with different rostro-caudal and medio-lateral inclinations were made through the same hole, in order to reach different locations. Kainic acid probes were inserted through rectangular trepanations (800 × 500 μm) made with microrazor-blades. Coordinates of all penetrations, including lesioning penetrations, were referred to the reference point of the equipment, and thus could be mathematically transformed to the coordinate system of a standard series of frontal sections, especially prepared for this bat species as a working brain atlas (T. Fenzl and A. Nixdorf, unpublished data).

Electrical microstimulation

For electrical microstimulation, parylene-coated tungsten electrodes (type TM33A20; WPI Inc., Sarasota, USA) with impedances between 1.9 and 2.2 MΩ were used. The electrodes were placed on the brain's surface and were lowered in steps of 100 or 200 μm using a piezoelectric micropositioner. The exposed electrode tips of the metal electrodes had diameters of 1 μm (sharpened tip). A sharpened tungsten wire mounted on an additional micromanipulator was inserted between the muscle fold and skull as indifferent electrode.

A Grass stimulator (type S 48; Grass, Quincy, USA) provided the electrical stimuli which were delivered as current stimuli by a photoelectric stimulus isolation unit (type PSI U6; Grass) to the electrodes at negative polarity. Typical stimuli consisted of bursts of rectangular pulses of 0.1 ms width at a frequency of 1 kHz. The bursts lasted 15 ms and were presented at repetition rates of 2, 3, 4 or 6 bursts/s. Stimuli currents ranged from 3 to 50 μA. Stimulus currents of 80 μA were used only for wide-range exploring within a particular area of interest.

Kainic acid stimulation

Kainic acid (α-Kainic acid, 98%; Diagnostic Chemicals Limited, Canada) was applied into PAG using microdialysis probes. The exposed tips of the homemade probes (R. Landgraf, Max Planck Institute of Psychiatry, Munich, Germany) had dimensions of 350 × 150 μm and a length of 1600 μm at the membrane. Within PLA, concentric microdialysis probes, purchased from Microbiotech (type MAB4.15.1.CA; Stockholm, Sweden) were used. These probes had tip diameters of 200 μm and 1000 μm of exposed membrane. Kainic acid, dissolved in 0.9% NaCl at a concentration of 144 pM, was pumped through the dialysis tubes at rates of 0.6, 1 or 1.2 μL/min, using a syringe pump (TSE-Systems, Bad Homburg, Germany) driving 2.5 mL Microlitre® syringes (Hamilton, Switzerland).

Current spread and chemical spread

The intracranial current spread for a stimulation current of 20 μA with a monopolar electrode is ≈70 μm when cell bodies are stimulated and ≈110 μm when myelinated fibres passing through (Ranck, 1975). In our experiments we found similar data. In particular stimulation sites of optimal vocal responses, a 100-μm dorsal or ventral shift of the stimulation electrode lead to a total lack of vocal answer when the stimulation current was held at the same level. Only with increasing stimulation current could a similar vocal answer be triggered at these remote areas.

McGeer *et al*. (1978) reported that diffusion of kainic acid to areas remote from the injection site cannot be seen when using small volumes and long injection times. For this study we used the microdialysed application of kainic acid to obtain these criteria because the only driving force to apply the substance is the concentration difference between the inner lumen of the probe and the tissue itself. Additionally, the stereotaxic device was used to place the microdialysis probes with an accuracy of better than 100 μm into the area probed for vocal response with electrical stimulation.

Monitoring of respiration rate and animal reaction

Respiration activity was measured with a small thermistor unit as part of a bridge circuit (homemade). The tiny thermistor probe was placed in front of the bat's nostril, the signals were amplified and stored on a Hameg storage scope (HM 205–3; Frankfurt a. M., Germany) for online monitoring of electrical and chemical induced reactions. During experiments the animal's reaction was continuously picked up by a TV camera (Teli, Tokyo, Japan) under infrared light illumination with an LED array (12 V/28 LED; Conrad Electronics, Germany), observed on a monitor (TC-800 E4D, Panasonic, Osaka, Japan) and recorded on a video recorder (NV-8200, Panasonic, Osaka, Japan). Body temperature of the animal was measured continuously with a temperature probe (Thermo clock Nr. 120145, Conrad Electronics) integrated into the sandwich holder.

Neuroanatomy

At the termination of experiments, the animals were lethally anaesthetized with Barbital® (16 mg/mL solution, 0.1 mL/10 g body weight) and transcardially perfused with 4% paraformaldehyd in 0.05 M phosphate buffer solution. Cryoprotection for freeze cutting was achieved by soaking the brains with 30% sucrose in 0.05 M phosphate buffer solution. The brains were embedded in egg yolk in a small Perspex chamber following a protocol (Schuller *et al*., 1986) which allows the alignment of the brain within the embedding block so that the ensuing section plane would optimally correspond to that of the reference brain sections of the brain atlas. The brains were freeze cut on a cryostat (Frigocut type 2700, Reichert-Jung, Germany) into 42-µm slices and generally three adjacent series were processed. Staining depended on the purpose of neuroanatomical processing and was performed following different protocols: Nissl stain or fibre stain (Gallyas, 1979)

Due to the standardized cutting procedure, data from individual brains could be well correlated to the sections of the reference brain, and thus made precise comparison of data between individual animals possible.

Sound recording and data analysis

The vocalizations of the bats were picked up with a 1/4-inch ultrasonic microphone (type 4135, Bruel & Kjaer, Darmstadt, Germany), amplified and sampled (250 kHz) with an A–D converter board (type CIO-DAS16/M1, Computer boards Inc., Mansfield, USA), and stored on a personal computer. Signals were recorded with the software 'Bat Sound' (Pettersson Electronic AB, Sweden) and/or with a self-programmed recording program based on Agilent-VEE (version 6.0 pro, Agilent, USA). Vocalizations were spectrally analysed and compared with known spontaneously uttered echolocation calls (echolocation calls were recorded in an acoustic chamber from a freely moving bat; Table 3) and communication calls of *P. discolor*, and correlations to the stimulation sites within the PAG and PLA were established. Types of calls were named after Kanwal (Kanwal *et al*., 1994); refer to Table 4 for details about the specifications of the calls we recorded. Only calls with a 20-dB peak amplitude above noise were used for evaluation. For analysis of the recorded vocalizations the software 'Bat Sound' (Petterson Electronic AB, Sweden) was used. Call length was evaluated using the envelopes of the vocalizations, peak amplitude of vocalizations was evaluated using the particular power spectrums and frequency shifts of vocalizations where measured within the spectograms.

Animal care

Principals of laboratory animal care were followed and experiments were conducted under the regulations of the current version of German Law and Animal Protection. Reference Government of Bavaria (Az. Reg. vs. Obb. 211-2531-37/98).

Results

Electrical stimulations and pharmacological stimulations in seven bats showed that the periaqueductal gray (PAG) and the paralemniscal area (PLA) have differentiated roles in the control of communication calls and echolocation calls. Stimulation of the PAG triggered diverse communication calls plus echolocation calls thus the PAG is involved in a vocal pathway that controls both types of calls. Stimulation of the PLA only triggered echolocation calls. The PLA seems to be exclusively involved in the control of this type of calls. These differences support our assumption of differential functional organizations in the vocal controlling pathways for communication calls and echolocation calls.

(1) Electrical stimulation in the PAG

The PAG around the mesencephalic aqueduct in *P. discolor* has a rostro-caudal extension of maximally 2500 µm, reaches laterally to a maximum of ≈1000 µm from the midline, and extends ≈2000 µm over the dorso-ventral dimension (brain atlas, T. Fenzl and A. Nixdorf) (Fig. 1B). The relative location and extension of the PAG in *P. discolor* is not different from that found in other mammals like rats (Van Bockstaele *et al*., 1991) or other bats (Suga *et al*., 1973). Electrical microstimulation in the PAG of *P. discolor* induces calls only in very restricted lateral and ventro-lateral areas within ≈700 µm caudal to the rostral margin of the PAG. Electrical microstimulations in the caudal half of the PAG did not trigger any vocalizations at all. Depending on the positive stimulation sites, different vocalization types can be distinguished in a single animal (Table 1; for classification see Table 4).

The recorded vocalizations can be classified as echolocation calls (Fig. 2A) or communication calls (Fig. 2B). Echolocation calls of *P. discolor* are typically frequency-modulated calls (FM) covering a frequency range between 45 and 100 kHz with the 3rd–5th harmonic (Rother & Schmidt, 1982), and constitute one class including 340 downward frequency-modulated calls (DFM; 46.4%) listed in Table 1. The 340 DFM calls could be subdivided (see Table 2) as follows: 88 of the calls (25.8%, type I) were typical short echolocation calls of *P. discolor* with a mean duration of 0.76 ± 0.28 ms (SD), whereas 58 calls (17%, type IV) were similar to echolocation calls with respect to their spectral composition, but showed overlap of the individual harmonics which cover wider frequency ranges and have a longer duration (mean 2.14 ± 0.91 ms). Thirty-four calls (10%, type III) were long DFM calls (mean 5.31 ± 2.25 ms) with the individual harmonics covering a frequency range narrower than in the echolocation calls. Twelve calls (3.52%, type II) showed curved downward modulated harmonics (mean 1.96 ± 0.68 ms SD). Of the remaining calls, 38 (11.2%) were presyllables of social calls, nine (2.6%) were fragments of calls and 101 (29.7%) could not be classified (see Table 1). Either peak amplitude was not 20 dB above noise or spectral composition was disturbed in such a way that correlation was not definite.

Besides the DFM calls, the communication calls were classified as follows (for all call abbreviations see Table 4): 102 vocalizations were broadband noise burst (BNB) calls; 23 vocalizations were

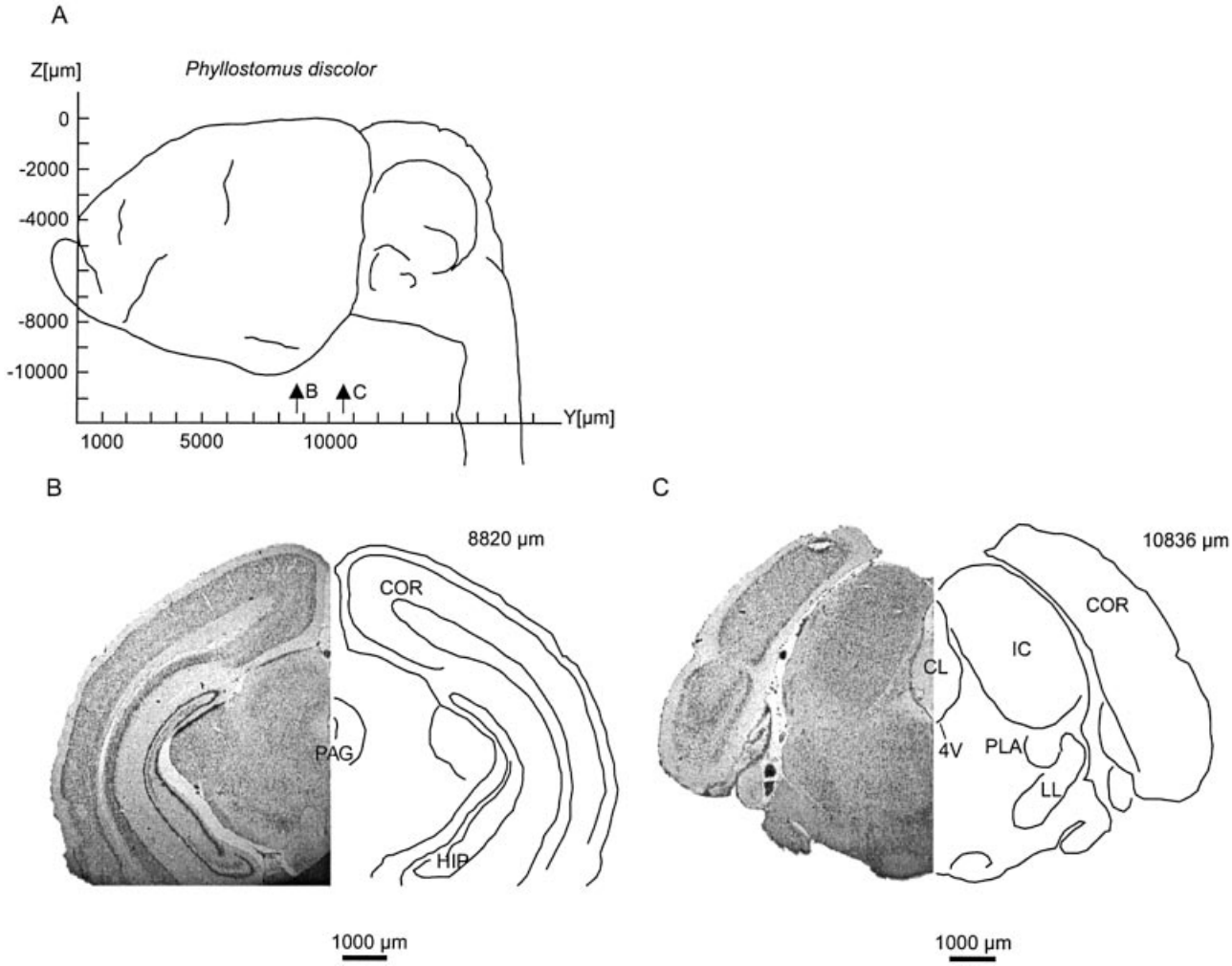

FIG. 1. (A) Sagittal view of the brain of *Phyllostomus discolor*. The arrows on the abscissa mark the two frontal sections at (B) 8820 µm and (C) 10836 µm. (B) Frontal section at 8820 µm with the rostral PAG region where vocalizations can be triggered. (C) Frontal section at 10836 µm showing the PLA represented by relatively large neurons in comparison to its surrounding areas. The sections in B and C are Nissl stained.

TABLE 1. Calls elicited by electrical microstimulation of the PAG

Vocalization type	Number of calls	Percentage of total (732) calls	Latency (ms) (mean ± SD)	Duration range (ms)	Threshold (µA)
DFM	340	46.4	29.5 ± 9.79	0.21–8.9	10
BNB	102	13.9	42.9 ± 15.95	39–196	10
qSFM	23	3.1	31.2 ± 6.98	40.1–216	20
DFM-WFM	13	1.8	56.1 ± 9.79	19–151.2	20
WFMl	8	1.1	111.6 ± 20.35	137–213	16
Not specified	246	33.6	–	–	–

Duration, minimum and maximum duration of all vocalizations of one type. Threshold, minimum value at which vocalization could be triggered. Not specified, unidentified vocalizations, either fragments of vocalizations or vocalizations with peak amplitude < 20 dB above noise.

quasi-sinusoidal frequency-modulated (qSFM) calls; 13 vocalizations were downward frequency-modulated–wrinkled frequency-modulated (DFM–WFM) calls; and 8 vocalizations were wrinkled frequency-modulated long (WFMl) calls. Two hundred and forty-six calls could not be classified at all. Classification was again not definite because peak amplitudes were < 20 dB above noise or because spectral composition was disturbed.

Stimulus-synchronized respiration can be triggered together with vocalizations or without eliciting vocalizations, e.g. at currents below the threshold for eliciting vocalization.

(2) Pharmacological stimulation in the PAG

In order to exclude stimulation of fibres passing through the stimulated area, kainic acid as a glutamate agonist was used to

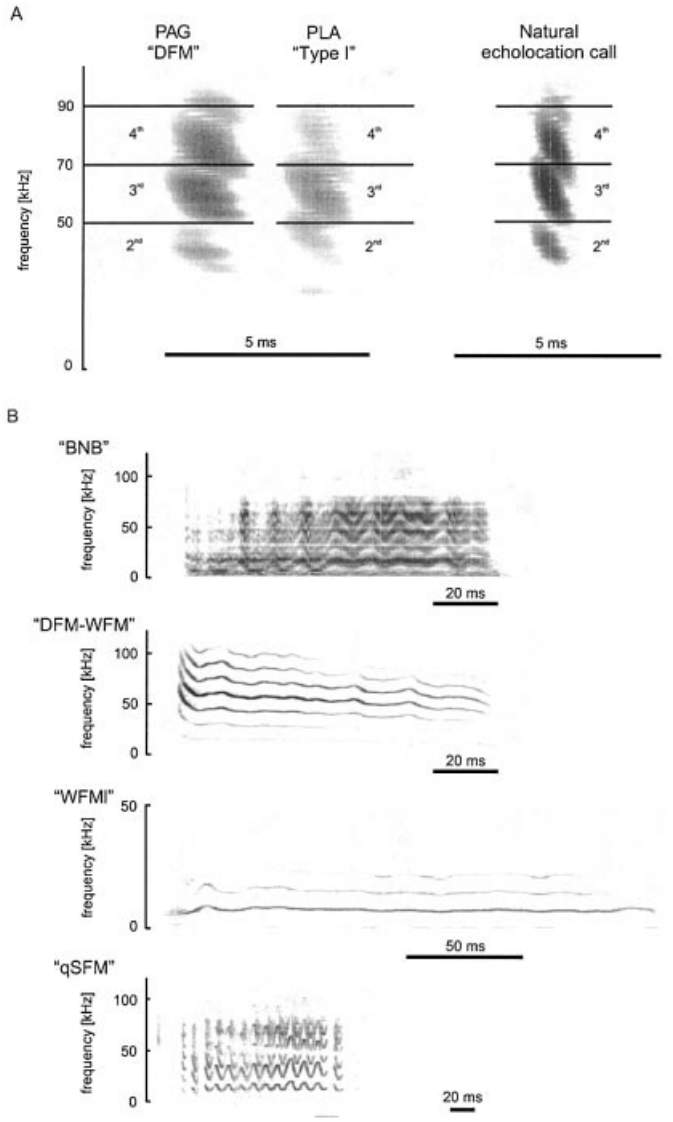

FIG. 2. (A) Two typical echolocation calls from *Phyllostomus discolor* elicited by stimulation of the PAG and PLA together with a natural echolocation call. The vocalization on the left is of vocalization type DFM (Table 1) and was elicited by stimulation of the PAG with a 30 μA current, the vocalization in the middle represents vocal type I (Table 5) and shows the answer of the PLA to a stimulation with 16 μA. The natural echolocation call on the right was recorded from a freely moving bat in an acoustic chamber; 2nd, 3rd and 4th refer to the individual harmonic. (B) Four communication calls. All four calls were triggered within the PAG. The upper call is classified as BNB because of its noisy character. The duration, intensity and proportion of noisy to sinusoidally modulated parts of the calls strongly depend on the stimulation current. The majority of the calls other than echolocation calls are of this type (see Table 1). This call together with the other three types of calls shown here were elicited in a single animal at stereotaxically different sites within the PAG.

activate neurons and elicit vocalizations in the PAG. Those regions in the PAG where electrical stimulation reproducibly triggered vocalizations other than echolocation calls at lowest thresholds were probed with kainic acid using microdialysis. Experiments with kainic acid were performed on one animal over a period of 10 consecutive days. The microdialysis probe was inserted into the PAG, and dialysis of kainic acid was turned on when the respiration rate was stable at the resting level of 3–4 cycles/s.

TABLE 2. Classification of DFM calls

Vocalization type	Number of calls	Percentage of total DFM calls	Duration (mean ± SD)
Type I	88	25.8	0.76 ± 0.28
Type II	12	3.5	1.96 ± 0.68
Type III	34	10	5.31 ± 2.25
Type IV	58	17	2.14 ± 0.91
Pre-syllables	38	11.2	–
Fragments of calls	9	2.6	–
Not specified	101	29.7	–

DFM calls are further subdivided into types I, II, III and IV calls; see Results, (1) Electrical stimulation in the PAG, for details.

TABLE 3. Spontaneously emitted echolocation calls

Number of natural calls	Duration (ms) (mean ± SD)	Starting frequency of third harmonic (kHz; mean ± SD)	Ending frequency of third harmonic (kHz; mean ± SD)
46	1.4 ± 0.46	78 ± 2.96	50 ± 1.05

Echolocation calls of a freely flying male *P. discolor*, recorded for comparison with electrically induced DFM calls shown in Tables 1 and 2.

TABLE 4. Description of sounds according to Kanwal *et al.* (1994)

Vocal type	Description
DFM	Downward frequency-modulated sound, including unidirectional downward sweeps with frequencies varying > 20% of the mean frequency of a single harmonic
BNB	Broadband noise burst with predominant bandwidth > 20% of the value of its centre frequency
qSFM	Quasi-sinusoidal frequency-modulated sounds where the rate of frequency modulation may vary and the sinusoidally frequency modulated pattern may be ramped upward or downward, or interrupted
DFM–WFM	Sounds with a wrinkled (irregular frequency modulation) part, initiated by a short DFM beginning
WFMl	Wrinkled (irregular frequency modulation) sounds of long duration

The classification given by Kanwal was used to categorize the elicited calls as five different types. The call lengths from our recorded vocalizations were evaluated using the envelopes of the vocalizations, peak amplitudes of vocalizations were evaluated using the particular power spectra and frequency shifts of vocalizations were measured within the spectograms.

Figure 3A and B show the timing of the experiment and the effect of kainic acid dialysed at a flow rate of 1.2 μL/min on respiration and vocalization rate. The insertion of the dialysis probe took 6 min, starting at minute 4 of the experiment. The removal of the probe took 2.5 min, starting at minute 41 of the experiment. The dialysis pump was turned on at minute 10 and turned off at minute 41 of the experiment. The gap in the respiration rate graph (Fig. 3B) at minute 4 is due to experimental procedure while inserting the probe. On kainic acid application the respiration rate rose from ≈ 4 cycles/s to a maximum of ≈ 13 cycles/s and dropped back to its resting rate again when dialysis was turned off. Clearly the increase in respiration rate preceded the increase in vocalization rate. Steady supply of kainic acid provoked a clear increase in the parameters vocal length, number of harmonics per call and call rate towards their respective maxima.

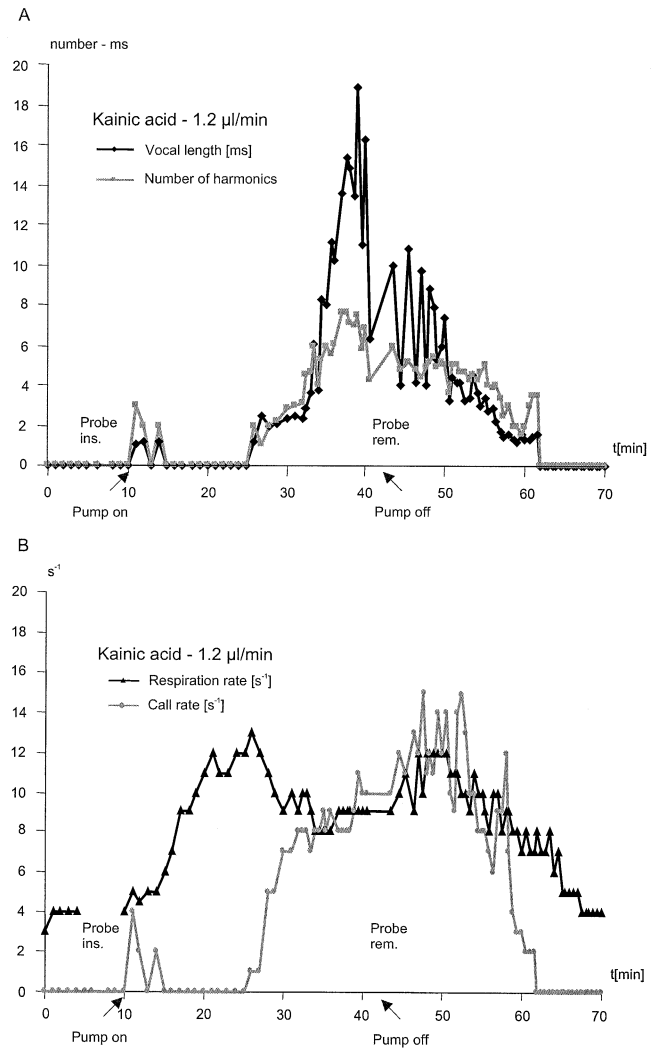

FIG. 3. (A) The effect of 1.2 µL/min microdialysed kainic acid on a vocally active PAG region. Both plots (A and B) originate from the same experimental run. The ordinate in plot A includes two dimensions: 'ms' refers to the vocal length per call, 'number' refers to the number of harmonics per call. In plot B, 's^{-1}' refers to exhalations per second and to call rate. Probe inserted (Probe ins.), Probe removed (Probe rem.). 'Pump on' and 'Pump off' are indicated with arrows in both plots. The whole kainic acid experiment took 20 days. The experiment plotted here was performed on day 15.

When vocalization rate and respiration rate showed a saturation at maximum response, the pump was turned off again.

Following probe removal, the vocalization stopped after 22 min and respiration rate dropped back to its resting frequency of ≈4 cycles/s, reaching control conditions. A recording of vocalizations at the time of maximum vocal length (Fig. 3, minute 35–40) is shown in Fig. 4, characterizing all vocalizations from the experiment shown in Fig. 3. Only one type of call was elicited with kainic acid. The spectral structure of this call characterized it as a communication call, especially when the call is compared with the single echolocation call marked with 'E' in Fig. 4. The control experiment with 0.9% NaCl (flow rate 1.2 µL/min) through the microdialysis probe at the same brain position did not show any effect on respiration, nor did it elicit vocalization.

The effect of kainic acid was dose-dependent and increasing dialysis rates of 0.6, 1 and 1.2 µL/min induced rises in vocal length and number of harmonics per call. Clearly the impact on the parameter 'vocal length' was strongest with 1.2 µL/min (Fig. 5A), decreasing with smaller doses.

Independent of the kainic acid dose applied, the leading gain in respiration rate with a delayed onset of vocalization could be determined in all experiments (data not shown).

Two days after completion of the experimental series, the animal was perfused and the different lesions localized (Fig. 6). The electrically induced lesion, produced by passing a negative current of 2 µA for 5 min through the stimulating electrode, was placed for verification of the stereotaxic position of the electrode and thus the dialysis probe (L1). Further lesion effects could be seen at the site where kainic acid was applied (L2). The multiple application of kainic acid over a period of 11 days could provoke lethal effects of kainic acid on neurons responsible for vocalizations.

(3) Electrical stimulation in the PLA

The region of the paralemniscal area (PLA) of *P. discolor* was first outlined on the brain atlas of *P. discolor* by analogy with the paralemniscal area as characterized neuroanatomically in *Rhinolophus rouxi* (Metzner, 1993) (S. Radtke-Schuller, Anat. Anst. LMU, Munich, Germany, brain atlas, unpublished data) and *Myotis montivagus* (Baron *et al*., 1996). In *P. discolor* the PLA is located medially, adjacent to the dorsal nucleus of the lateral lemniscus, and contains neurons larger than those in surrounding areas. Dorsally the PLA is bordered by the ventral margin of the inferior colliculus. The overall rostro-caudal extension of the PLA is ≈1200 µm and the medio-lateral dimensions are ≈750–800 µm at its maximum (Fig. 1C). Vocalizations could be triggered in a restricted area outlined in Fig. 7 without, or with only minor, pinna and/or body movements accompanying the vocalizations at low stimulation currents (minimum threshold was 3.4 µA). An increase in stimulation current typically caused body movements and arousal of the animal due to an enlarged radius of current spread. At stimulation sites with higher stimulus current thresholds, between 20 and 50 µA, elicited vocalizations were therefore in general accompanied by body movements and by arousal of the animal. Body movements were often accompanied by lasting nonspecific vocalizations due to the commotion of the animal. Detection of echolocation calls is impossible within these vocalizations of high intensity. Licking in addition to vocalization could be triggered at the medial and lateral margins of the caudal end of the PLA.

The elicited vocalizations at the optimum stimulation sites, with stimulation currents < 15 µA, were always DFM calls and never communication calls as elicited by stimulation of the PAG. All 984 recorded calls are listed in Table 5. Of these calls, 71.1% were DFM calls; 283 calls, also indicated as DFM calls, are not included as their peak amplitude was < 20 dB above noise.

Calls identified as DFM calls may differ in duration and frequency ranges when considering the starting frequency of the third harmonic. Straight downward modulated harmonics are present in vocalization types I, II, III and IV, whereas only vocalization type V displays a slightly curved course of frequency modulation. The typical DFM calls from PAG stimulation and from PLA stimulation in Fig. 2 show no apparent differences in spectral structure. Both echolocation calls have almost identical duration and their corresponding harmonics cover the same frequency ranges. As in the PAG, the respiration rate could also be influenced by stimulating the PLA. Of the 484 recorded PLA stimulation runs, 44% show correlation of the respiration rate with the stimulating frequency. In 17% of stimulation runs the amplitude of the respiration signal increased, which suggests a stronger activity of the expiratory muscles due to stimulation of the

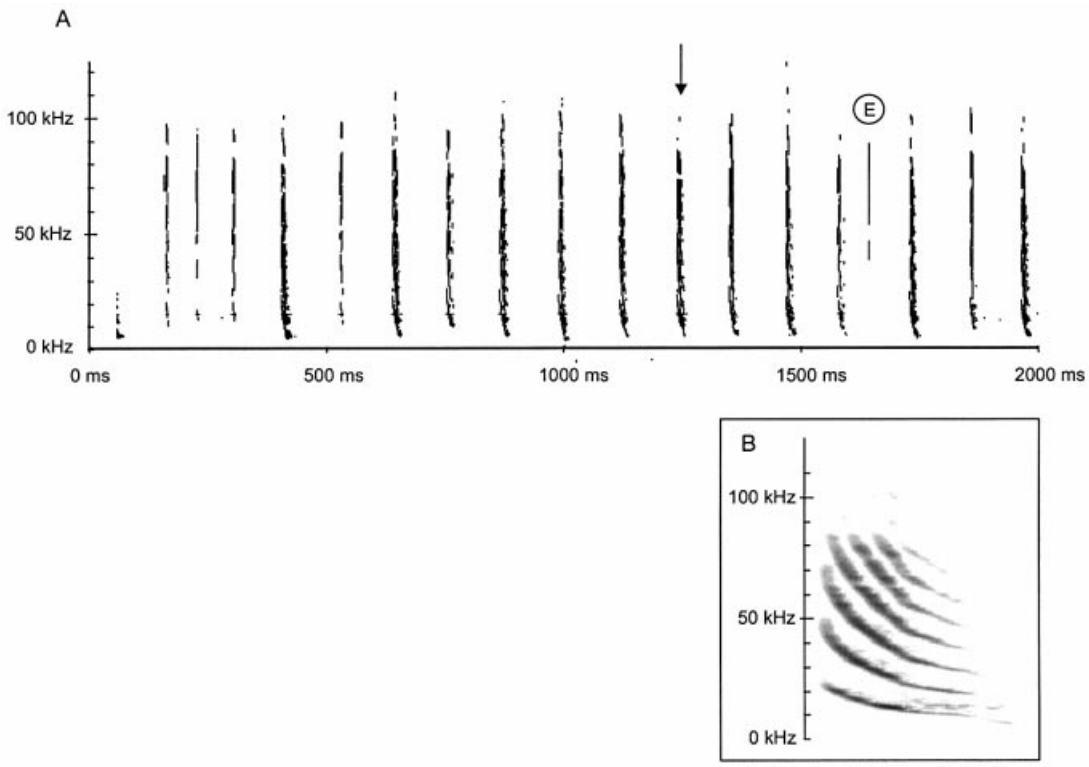

FIG. 4. (A) Microdialysed kainic acid triggered vocalization in the PAG. Twenty-nine minutes after the dialysis pump was turned on (see Fig. 3 for details), 1.2 μL/min kainic acid caused vocalizations as shown in A. Among the 17 calls with nearly identical spectral and harmonic structure, one echolocation call is emitted, marked with E. (B) Spectral structure of a single call (duration 15 ms), taken from plot A and marked with an arrow.

PLA, independent of whether the respiration signal was correlated with the electrical stimulus.

(4) Pharmacological stimulation in the PLA

The PLA was also probed with kainic acid to exclude fibres of passage as a possible source of vocal response when stimulated by electrical microstimulation. Because a microdialysis rate of 1.2 μL/min showed best results in the PAG, it was also used in the PLA region. This experiment was performed with one animal on one day and is shown in Fig. 8.

The probe was inserted at the PLA coordinates determined previously with electrical microstimulation. The insertion of the dialysis probe took 2 min starting with minute 2.5 of the experiment. The temporary drop in respiration rate down to 1 cycles/s after insertion of the probe may be a distortion rather than the resting respiration rate of the bat. The dialysis pump was turned on at minute 7.5 and turned off at minute 20 of the experiment. Removal of the probe took 1.5 min starting with minute 22.5 of the experiment. Kainic acid induced a relatively quick onset of respiration rate increase which preceded the onset of vocal activity in the PLA. Because the effect of kainic acid on neurons can be seen within the first third of the experiment and respiration rate as well as vocalization were maintained throughout the rest of the experiment until the bat stopped vocalizing, only 40 min of the 130-min experiment are plotted.

All kainic acid-induced vocalizations from the PLA were DFM calls. Figure 9 shows a record of pharmacologically triggered vocalizations. The calls form clusters of three, separated by distinct intervals between the groups of calls with a mean call interval of 31.66 ± 3.57 ms and a mean group interval of 54.53 ± 7.68 ms.

This grouping was encountered in all 46 recordings (Table 6) of the 40 min experimental run shown in Fig. 8. The kainic acid-induced vocalizations were of type II (refer to Table 5), as shown in Fig. 10.

Discussion

This study has demonstrated that the control of different types of vocalizations may rely on different neural substrates.

It is well established that the PAG plays a crucial role in the production of communication calls (Jürgens, 2002). Our examinations of the PAG in the bat *Phyllostomus discolor* using electrical and pharmacological stimuli support these findings. Four different types of communication calls could be elicited by electrical stimulation. Additionally, echolocation calls could be triggered in the PAG. This supports the statement that the PAG is involved in vocal pathways that control both echolocation and communication calls. In contrast, electrical stimulation of the PLA could trigger only echolocation calls, as has been shown in other bats (Schuller & Radtke-Schuller, 1990; Schuller, Fischer *et al*., 1997). The PAG and the PLA therefore may interact differentially with the pathway for vocalization.

Periaqueductal gray in Phyllostomus discolor

The periaqueductal gray of the midbrain plays an important role in vocal control of several species (Jürgens, 1998). A lesion in the PAG can lead to complete mutism. Also, in humans, ischemic lesions in this region cause irreversible mutism, although language comprehension functions and nonverbal expression capacity can be preserved (Esposito *et al*., 1999). Electrical stimulation in the PAG

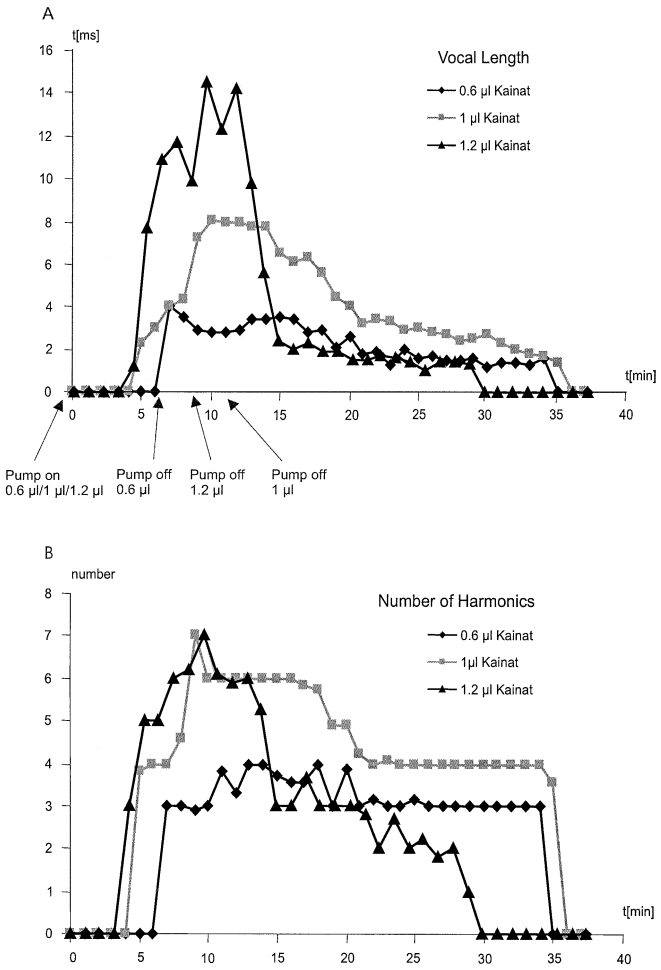

FIG. 5. The effect on the vocalization behaviour of kainic acid (Kainat) applied in the same area with different doses. (A) Vocal length of individual calls each minute. Clearly for 0.6 µL/min dialysed kainic acid the response was weak, rising with 1 µL/min kainic acid. Maximum values were reached with a dose of 1.2 µL/min dialysed kainic acid (pump on for all three experiments at minute 0). In all experiments the gain in respiration rate preceded the onset of vocalization (not shown here). (B) Gain in the number of harmonics from the same three experiments shown in A. Note that the application time for 1 µL kainic acid is longer than for 1.2 µL kainic acid, thus leading to a slightly extended vocalization period for 1 µL kainic acid (plot A). The whole kainic acid experiment took 20 days. The 0.6- and 1.2-µL experiments were performed on day 7, the 1-µL experiment on day 5. At day 20 an additional control experiment with 0.9% NaCl was performed after the animal was tested positive with electrical microstimulation on the explored sites.

triggers vocalizations in different mammalian (Kirzinger & Jürgens, 1991); (Zhang *et al*., 1994) and nonmammalian (Kennedy, 1975) species.

The location and anatomical structure of the PAG in *P. discolor* is similar to that of other mammals (Bandler *et al*., 1991; Van Bockstaele *et al*., 1991). The vocally active sites in the PAG of *P. discolor* are restricted to lateral and ventro-lateral areas within the rostral half of the PAG whereas the entire caudal half of the PAG is vocally not active.

Stimulation in the lateral margins of the PAG in the bat *Rhinolophus rouxi* elicit distorted vocalizations accompanied by various degrees of arousal of the animal (Schuller & Radtke-Schuller, 1990), but a complete scan of the entire PAG with electrical stimulation has not been performed in this species.

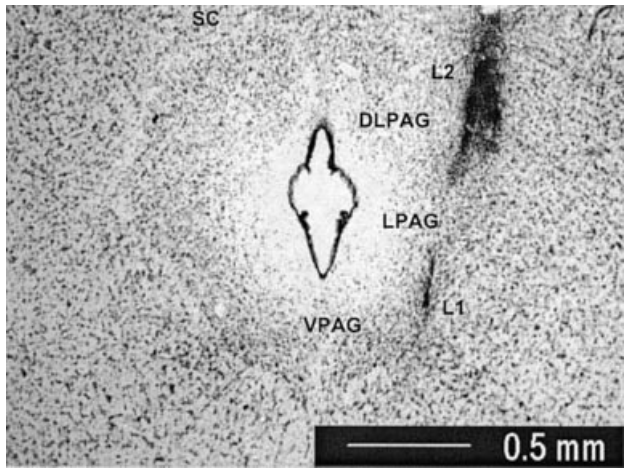

FIG. 6. A kainic acid-induced lesion (L2), where vocalizations can be triggered, is clearly visible within the lateral PAG (LPAG). Additionally, an electrically induced lesion (L1) for exact measurement of stereotaxic data in the ventrolateral PAG can be seen. DLPAG, dorso-lateral PAG; VPAG, ventral PAG. Kainic acid-induced vocalizations as shown in Fig. 4 were triggered at the L2 area.

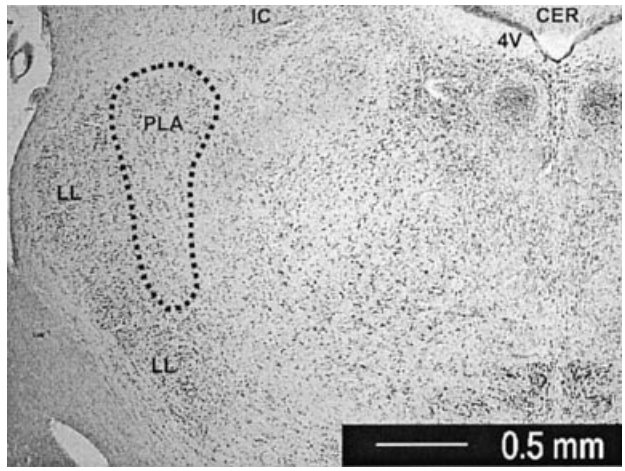

FIG. 7. The PLA in the brain of *Phyllostomus discolor*. The region where electrical stimulation triggers vocalizations is outlined in this Nissl-stained section. 4V, 4th ventricle; CER, cerebellum; IC, inferior colliculus; LL, lateral lemniscus; PLA, paralemniscal tegmental area.

Ennis *et al*. (1997) traced the connection of the PAG to the nucleus ambiguus region in rats by retrograde labelling of PAG neurons. Labeled neurons are found laterally in the rostral two thirds and ventrolaterally also through the caudal third of the PAG. Larson & Kistler (1984) report on cellular activity connected to vocalization only in a small area in the dorsal part of the PAG of monkeys. Jürgens (1994), however, mentions that vocalization-eliciting sites are not restricted to a specific subarea of the PAG across different species. According to his studies in the squirrel monkey, such an extensive distribution is only found if the data from several animals are pooled. In the single animal, the vocalization-eliciting area in the monkey seems to be as restricted (Jürgens, 1994) as in our study with *P. discolor*.

(1) Electrical stimulation in the PAG

The PAG-induced vocalizations of *P. discolor* can be subdivided into echolocation calls on the one hand and natural-sounding

TABLE 5. Calls elicited by electrical microstimulation of the PLA

Vocalization type	Number of calls recorded	Percentage of total 984 calls	Duration (ms, mean ± SD)	Starting frequency of third harmonic (kHz, mean ± SD)	Ending frequency of third harmonic (kHz, mean ± SD)	Latency (ms, mean ± SD)
I	343	34.8	1.53 ± 0.61	72 ± 4.45	53 ± 3.33	33 ± 11.87
II	36	3.6	3.45 ± 1.07	85 ± 1.84	53 ± 2.78	27 ± 1.26
III	290	29.5	2.38 ± 0.83	78 ± 4.06	53 ± 3.59	32 ± 12.16
IV	28	2.8	2.12 ± 0.68	65 ± 3.74	48 ± 4.00	35 ± 2.09
V	4	0.4	3.36 ± 1.60	73 ± 1.41	54 ± 7.78	34 ± 1.56
Not specified	283	28.9	–	–	–	–

All types of vocalizations (types I–V and not specified) represent DFM calls. 'Not specified' (unidentified) comprises all calls with a peak amplitude < 20 dB above noise.

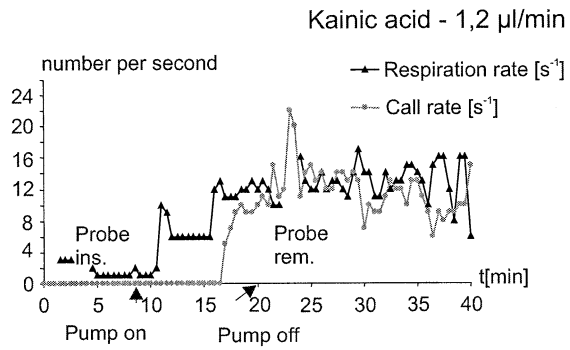

FIG. 8. The effect of microdialysed 1.2 µL/min kainic acid on the vocally active PLA region. With respect to the temporal development of respiration rate and call rate the results are similar to those in kainic acid PAG experiments. Probe inserted (Probe ins.), Probe removed (Probe rem.). 'Pump on' and 'pump off' are indicated with arrows.

communication calls on the other. It has been shown that echolocation calls can be elicited by stimulation of the lateral part of the PAG in *Myotis spec*. and without specific localization in the PAG of *Pteronotus parnellii*, *P. suapurensis* and *Noctilio leporinus* (Suga *et al*., 1973).

In their spectral composition, the calls elicited in *P. discolor* of type DFM (Table 1) closely resemble the natural echolocation calls, whereas their duration shows a wider spread than described for spontaneously emitted calls (Rother & Schmidt, 1982). The duration, bandwidth and interpulse intervals of echolocation calls as published for this species may not reflect the real potential of these parameters because they were recorded in the laboratory (see Discussion, electrical stimulation in the region of the PLA).

Subtypes I and IV of DFM calls (Table 2) correspond closest to echolocation calls. Subtypes II and III (Table 2) show a curved pattern and long calls up to ≈ 9 ms occur. The calls of type II and III could also correspond to what Esser & Pistohl (1998) describe as social calls of a possibly nonagonistic behaviour. The BNB calls (Table 1) roughly resemble in mean duration and frequency range a threat call with a noisy character and an aggressive call with a sinusoidal character that have been recorded from *P. discolor* in behavioural experiments (Esser & Pistohl, 1998). It is noticeable, however, that the stimulation experiments did not result in the production of all communication calls described by Esser & Pistohl (1998). The majority of PAG-induced call types in the cat and squirrel monkey are of agonistic kind (Jürgens, 1994; Jürgens, 2002) and Table 1 reveals equivalent findings because the majority of elicited calls other than DFM calls are BNB calls. Jürgens (2002) concludes that the PAG has a basic gating function relating to voluntary as well as unconditioned vocal reactions. Because the PAG belongs to the limbic system, a network responsible for the control of motivation, the constraints of the experiment could be directly reflected in an agonistic disposition of the animal and its appertaining types of vocalizations, controlled by the limbic system and the PAG as part of it. However, all types of calls elicited by stimulation of the PAG should be reflected in the vocalization repertoire of the animal. Such an equation between elicited calls and natural calls is supported by Jürgens. He mentions that PAG-induced vocalizations generally resemble natural calls (Jürgens, 1994). Based on this, not only the DFM but also all other types of calls elicited by stimulation of the PAG should conform with natural calls, even though we cannot establish a connection between all of the communication calls elicited and a particular behaviour.

The locations of specific vocalization-eliciting sites for different call types in the PAG overlap to a large degree and are not organized in distinct contiguous clusters for individual call types in the PAG (Jürgens, 1994). Therefore different types of calls, possibly embedded in the same behaviour of the animal, may be evoked in a single area representing the behavioural context.

Such overlap of sites with elicited vocalizations of different characteristics can also be found in *P. discolor*. Stimulation at the same coordinates of particular PAG areas in subsequent penetrations may result in the emission of different call types. Call types can also change when the stimulation current is increased.

However, most of the vocalizations are consistently elicited over even long periods and at relatively short latencies in the PAG (Table 1). Much longer latencies of the response would be expected if vocalizations were induced as an integral part of a behaviour. This argues against the concept that vocalizations were secondary reactions to motivational changes due to stimulation (Jürgens, 1994), or the experiment itself. Our results support conclusions from findings in the squirrel monkey (Jürgens, 1994) that vocalizations triggered in the PAG are not directly controlled by behavioural activation and that the PAG has a rather direct access to the descending vocalization pathway.

(2) Pharmacological stimulation in the PAG

Kainic acid, one of the most powerful glutamate analogues that has been studied for excitatory potential (Olney, 1978), induced calls which differed from those electrically elicited at the same site in the PAG and which were different from echolocation calls. This could be due to the overlap of vocalization-eliciting sites within the PAG, or a consequence of the much larger diameter of the dialysis probes, thus delivering the kainic acid to a much larger area at the same time.

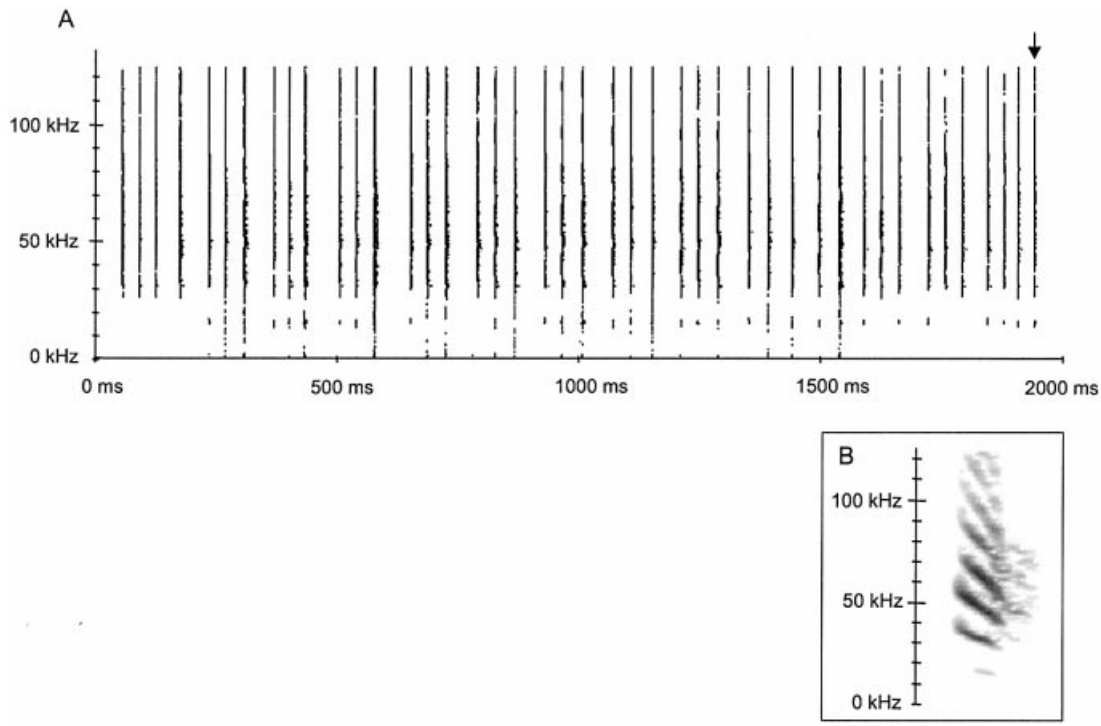

FIG. 9. Microdialysed kainic acid triggered vocalization in the PLA. (A) A vocalization train 15.5 min after the dialysis pump was turned on. Calls are of DFM type, and typically clustered in groups of three. (B) A single echolocation call (duration 3.5 ms) taken from the recording in A, indicated by the arrow.

TABLE 6. List of groupings within all 46 recordings from the experiment shown in Fig. 8

	Grouping (number of individual calls per syllable)							
	1/2	2	2/3	3	3/4	2/3/4	8	No grouping
Number of recordings	11	19	10	1	1	1	1	2

Grouping refers to the total number of individual calls per syllable: 1/2, single calls or groups of two calls within one syllable; 2, groups of two calls only within one syllable; 2/3, groups of two or three calls only within one syllable and so on.

Irrespective of this it must be supposed that vocalizations can only be triggered by exceeding a particular minimum concentration of kainic acid within the brain tissue. At this point the individual calls show a very constant duration (Fig. 4) while at the onset and end of vocalization the duration of the calls is decreased. Concentration increase and concentration decrease effect in the application area can be seen at the beginning and the end of each kainic acid experiment. The delayed onset and slow rise of vocalization rate can be explained with an increase towards the concentration required for the stimulation of vocalization areas. In contrast to pressure injection of kainic acid, the use of microdialysis allows only a very slow gain of concentration within the tissue because the only driving force to apply the substance is the concentration difference between the inner lumen of the probe and the tissue itself. The delayed and relatively long fading of vocalization can be explained with slow dilution of kainic acid within the brain tissue.

An inhibitory control of vocalization in the PAG is exerted by GABA (Jürgens & Lu, 1993). GABAergic afferents seem to have a tonic inhibitory control on the PAG vocalization mechanism (Lu & Jürgens, 1993), and Sakamoto *et al*. (1993) discuss how GABAergic inhibition regulates the excitability of the cells within the ventrolateral PAG, which exerts an excitatory effect on the lower brain structures that produce vocalization. Additionally it has been shown repeatedly that kainic acid does depress GABA-mediated inhibition in CA1 pyramidal cells (Ben Ari & Cossart, 2000). Even though the mechanisms and the physiological relevance of this effect is unclear, kainic acid-induced depression of inhibition should also be discussed as an additional explanation for the slow attenuation of vocalizations after kainic acid application to PAG neurons.

The response of the respiration rate to kainic acid is somewhat surprising, because all kainic acid experiments show that the onset of increasing respiration rates always precedes the onset of vocalizations itself (Fig. 3), whereas after ending of vocalizations the respiration shows a delayed response towards decreasing kainic acid concentration. It can be assumed that the respiration rate increase is not a consequence of triggering vocalization but rather a separately modulated mechanism. Behaviours such as vocalizations, different stages of defence (Fanselow, 1991), defence freezing and flight reactions (Morgan *et al*., 1998), reproductive behaviour (Ogawa *et al*., 1991) and hypotension plus decreased vasoconstrictor tone to allow

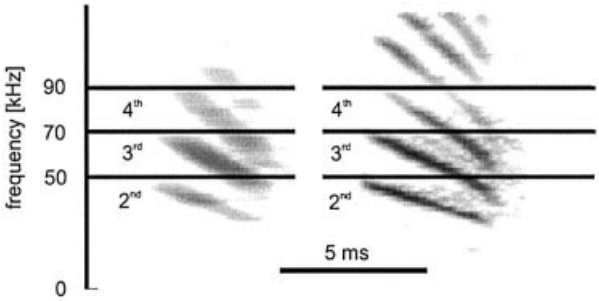

FIG. 10. Two DFM calls of *Phyllostomus discolor* elicited by stimulation of the PLA. The left call (taken from vocal type II in Table 5) in the plot is the answer to a stimulation with 14 μA while the right call represents the answer to 1.2 μL/min kainic acid. Apart from the small difference of the durations the two calls are spectrally almost identical. The starting and ending frequencies in the 2nd to 4th harmonic are the same and the multi harmonic downward frequency modulated pattern is similar. The recording is limited to 125 kHz and therefore crops the upper frequencies in the call on the right side.

the animal to rest and recuperate efficiently (Carrive, 1991), can be induced or modulated by PAG stimulation. An adaptation of respiration rate with respect to the behaviour being performed can be assumed. The PAG therefore could serve as an integrator between a particular behaviour and the adequate respiration rate.

The respiration rate can also be influenced by electrical stimulation in vocally active areas without eliciting vocalizations. It has been shown in the cat that respiratory-related cells can be found in the PAG (Ni *et al*., 1990). The authors argue that a subset of PAG cells may play a role in state-related respiratory patterning. In addition, it has been suggested that different constellations of activated PAG cells during vocalizations may lead to varying patterns of muscle activities and thereby assist in determining the quantitative and qualitative aspects of vocalization (Larson, 1991). In the rat, an electrophysiological link between the PAG and respiratory neurons of the nucleus tractus solitarius was shown (Huang *et al*., 2000), and the authors suggest an integral role of the PAG as a cardiorespiratory modulator to a number of behavioural responses initiated in other areas of the PAG. In combination with our findings, an independent PAG control of respiration commanded through different behaviours embedded in the PAG could be conceivable.

The region of the paralemniscal area in Phyllostomus discolor

The vocally active paralemniscal tegmental area in the bats *Rhinolophus rouxi* and *Pteronotus p. parnellii* is located in a restricted tegmental region rostral and medial to the dorsal nucleus of the lateral lemniscus (Schuller & Radtke-Schuller, 1990; Schuller *et al*., 1997). The paralemniscal area in the brain of *P. discolor* can be found at a similar position, but the vocally active sites extend ≈ 400–500 μm more ventrally.

Other than in bats, the paralemniscal area has rarely been investigated for its integration in the production of vocalizations. Only in the squirrel monkey the ventral end of the paralemniscal area has been found to be involved in vocalizations (Jürgens, 2000).

(1) Electrical stimulation in the region of the PLA

The electrical microstimulation and pharmacological stimulation in the part of the paralemniscal area marked in Fig. 7 yield results similar to those found in *Rhinolophus rouxi* (Pillat & Schuller, 1998). All calls elicited by stimulation of the PLA are DFM calls (Table 5) showing the typical harmonic structure of a *Phyllostomid*-like echolocation call (Rother & Schmidt, 1982; Esser & Daucher, 1996) with small variations. The frequency ranges of all five types of calls show similar values for their ending frequencies while the starting frequencies show more variation, which reflects the natural variation of echolocation calls. For a longer call the bat either can sweep through a broader frequency range by raising the starting frequency of the harmonics, or it can prolong the call itself without changing the bandwidth. When approaching a target, typically the bandwidth of the signal increases while the sound duration decreases (Kalko & Schnitzler, 1998). This could explain the variations in duration and frequency shifts shown in Table 5 for the DFM calls. Furthermore, the echolocation calls of free moving bats in the field have longer durations, longer interpulse intervals and a greater variability in bandwidth than signals recorded in the laboratory (Surlykke & Moss, 2000).

The very short and stable response latencies (Table 5) and low thresholds in *P. discolor*, which both argue for a direct access of the PLA to the descending vocal pathway for echolocation calls, are also found in *Rhinolophus rouxi* and *Pteronotus p. parnellii* (Schuller & Radtke-Schuller, 1990; Schuller *et al*., 1997). In addition, pinna movements are elicited with thresholds equal to or lower than triggering vocalization in these species, as well as in *P. discolor*. In the present work, experiments confirm these statements.

(2) Pharmacological stimulation in the region of the PLA

Different from findings in the PAG, kainic acid induced the same echolocation call as electrical microstimulation at similar stimulation sites in the PLA (Fig. 10). This was independent of the much larger diameter of the dialysis probe and thus a much larger area of stimulation.

However, kainic acid induces grouping of calls (Fig. 9 and Table 6), which is not found in electrically induced calls with a one-to-one stimulus–call relationship. Such grouping of echolocation calls in clusters is also found in spontaneously vocalizing *P. discolor*, when stationary or flying in an arena (Rother & Schmidt, 1982). As kainic acid stimulation of the PLA leads to emission of echolocation calls in a naturally occurring constellation, the PLA might represent an area influencing this behaviour. The grouping of vocalizations in *P. discolor* is paralleled by a corresponding change in respiration rate and does not represent multiple vocalizations. Multiple vocalizations within exhalation have been triggered with electrical microstimulation in *Rhinolophus rouxi* (Schuller & Radtke-Schuller, 1990) in the nucleus cuneiformis and the adjacent area ventrally.

The respiration rate onset and call rate onset after kainic acid application is similar in PAG and PLA experiments (Figs 3 and 8), and therefore may access control of respiration similarly via a final common pathway. Possible stages of convergence of the postulated pathways towards a final common pathway could be the bilaterally organized dorsal and ventral groups of respiratory neurons (Bianchi *et al*., 1995; Richter & Spyer, 2001) within the lower brainstem. Here the pre-Bötzinger complex within the ventral groups of respiratory neurons contains all classes of neurons important for respiratory rhythm generation (Connelly *et al*., 1992; Schwarzacher *et al*., 1995). It cannot be excluded that the two pathways also converge in this respect above the level of the medulla, and neurons, responsible for respiration muscle patterns inside the PAG (Davis *et al*., 1996), could get input from the PLA, influencing respiration patterns.

Latencies for echolocation calls

The latencies for echolocation calls elicited from the PAG and PLA appear similar (Tables 1 and 5). These measured latencies represent

the composition of several time components, namely the onset of the stimulus, the latency of interneurons and premotoric neurons and the latency of the motor neurons itself. Rübsamen showed that, at the level of the nucleus retroambiguus, motor neurons answer between 10 and 15 ms prior to the onset of spontaneously emitted vocalizations (Rübsamen & Betz, 1986), so the given latencies for elicited vocalizations are consequently a summation of individual latencies given for each step along the vocal pathways. An exact calculation of these individual latencies is impossible because we do not know all components of the pathways and thus we have not discussed different pathways on the basis of latencies.

Summary and conclusion

Electrical microstimulation in the PAG triggers communication calls and echolocation calls. The PAG, involved in control of social calls as well as of echolocation calls, constitutes part of the vocal pathway. Assuming that echolocation calls developed from communication calls (Gould, 1971; Gould, 1977; Gould, 1983; Fenton, 1984), the PAG as an integrator for particular behaviours and its required vocalizations could possibly embed both types of vocalizations.

The PLA, however, seems to be exclusively involved in the control of echolocation calls, and this echolocation pathway could be a special adaptation required for the fine contour of echolocation calls.

Descending vocalization control for communication calls and echolocation calls seem to differ in their functional organization. PAG and PLA therefore may interact differently with the final common pathway for vocalization.

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Abbreviations

BNB, broadband noise burst; DFM, downward frequency-modulated; DFM–WFM, downward frequency-modulated–wrinkled frequency-modulated; PLA, paralemniscal tegmental area; PAG, periaqueductal gray; qSFM, quasi-sinusoidal frequency-modulated; WFMl, wrinkled frequency-modulated long.

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Paper 2

Echolocation calls and communication calls are processed differentially in the brainstem of the bat *Phyllostomus discolor.*

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Abstract

Vocalizations of bats can be separated in two different classes, namely echolocation calls and communication calls. Independent from this differentiation the production of all types of calls must have access to the same pool of motoneurons responsible for vocalizations. In the periaqueductal gray matter (PAG) communication calls and echolocation calls can be elicited, whereas in the paralemniscal area (PLA) only echolocation calls can be elicited. Both structures show low current thresholds for triggering natural vocalizations without habituation to the stimuli even for long stimulation periods meaning that these structures have relative direct access to the final common pathway for vocalization. Reversible blockades of the PLA prevent periaqueductally induced echolocation calls while communication calls elicited in the same area are not affected. Tracer studies were executed to obtain insight into neuroanatomical connections between PLA and PAG in respect to their access to the final common pathway for vocalization. It is concluded that neural control of echolocation and communication calls is differentially organized, before it shares the final common pathway for vocalization.

Introduction

Motoneurons controlling the larynx, the vocal tract and the respiratory muscles are accessed via interneurons from the nucleus ambiguus/retroambiguus complex (Zhang et al., 1995) through the final common pathway for vocalization. One major subcortical structure for the control of vocalizations above the level of the medulla is the periaqueductal gray matter (PAG). Here social calls can be elicited in several mammals like Squirrel monkey (Lu & Jürgens, 1993), Rhesus monkey (Larson & Kistler, 1984), rat (Yajima et al., 1980), guinea pig (Martin, 1976), gibbon (Apfelbach, 1972) and bat (Fenzl & Schuller, 2003; Valentine et al., 2002) While in most areas, where vocalizations can be elicited, long-lasting stimulation causes a vanishing of the vocal reaction, the PAG is one of the very few regions where natural communication calls can be elicited without changes in the emotional state of the animal and without habituation to the stimulus, even over extended stimulation periods (Jürgens, 1994).

In bats stimulating the PAG evokes natural echolocation calls (Suga et al., 1973, Suga & Yajima, 1988) in addition to communication calls without motor concomitants (except adequate ear and nose leaf movements) also without habituation to the stimulus (Fenzl & Schuller, 2002). The PAG in bats is therefore involved in control of both types of vocalization, social calls as well as echolocation calls.

Phyllostomus discolor uses a rich repertoire of about 20 different social calls (Esser & Pistohl, 1998) and very stereotyped frequency modulated echolocation calls (Rother & Schmidt, 1982). Echolocation calls cannot only be triggered in the PAG but in a variety of brainstem areas (Schuller & Radtke-Schuller, 1990). Of all these areas, in *Rhinolophus rouxi* the lowest current thresholds and short latencies for eliciting echolocation calls are found in the paralemniscal area (PLA) (Schuller & Radtke-Schuller, 1990). The PLA seems to posses this property in bats in general, as also been demonstrated in *Pteronotus p. parnellii* (Schuller t al., 1997) as well as in the bat used in this study, *Phyllostomus discolor* (Fenzl & Schuller, 2002; Nixdorf, 2003). As the PLA displays the same characteristics as the PAG, in terms of microstimulation parameters for eliciting vocalizations, it can be assumed that the PLA also must have access to neural networks for the production of vocalizations similar to the PAG.

A vocal pathway from the PAG directly to the nucleus ambiguous/retroambiguus complex as described in the literature, is therefore not sufficient to explain findings on the PLA and other lower brainstem structures involved in the control of echolocation calls in bats. Rather a parallel organization of the descending control system for vocalizations can be assumed at this level, and it is of interest whether this organization is a general mammalian feature.

The present study investigates whether the PAG and the PLA interact during the PAG-induced production of communication calls and/or echolocation calls. Differences of the effect of reversible blockades of the PLA on triggering communication calls and echolocation calls in the PAG, respectively, should provide arguments for or against the hypothesis that vocal pathways for the production of communication calls and echolocation calls are in part separately organized.

Material and Methods

Experimental design

The experiments were designed to explore a potentially parallel organization of the neural pathways for vocal control of communication calls and/or orientation calls. Electrical stimulation within the PAG triggers either echolocation calls or particular types of communication calls, depending on the stimulation site. Two stereotaxically implanted electrodes in the PAG yielded either echolocation calls or communication calls upon electrical micro-stimulation. Concurrent blockades of one or two PLA sites should either block the production of PAG induced communication calls, echolocation calls or both, if a vocal pathway for vocalization from the PAG to the final common pathway for vocalization involves PLA sites.

Two male neotropical bats, *Phyllostomus discolor*, were used for the blockade studies and additionally ten male bats where used for tracer studies. All animals originated from a pure male colony, separated from a mixed breeding colony. During the experiments the particular animal under investigation was kept separate from the male colony but under the same semi-natural conditions (light cycle 12 h/12 h, 65 % - 70 % humidity, 25 - 30 degree centigrade).

Surgical preparation of the animals was done under Isoflurane (CuraMED Pharma, Karlsruhe, Germany) anesthesia with an initial dose of 4% Isoflurane and a sustained dose of 3.4 – 4% to maintain the anesthetic state of the animal. Skin and muscles overlying the skull were locally anaesthetized with 2% Xylocain® (Astia, Germany).

The skin and muscles overlaying the skull were cut rostro-caudally along the midline, reflected to the sides and kept in place using dental sponge (Gelastypt®, Hoechst, Germany). Minor bleedings were stanched with the coargulant Orbat® (lege artis Pharma, Germany).

A small holding tube to allow later fixation of the head in a stereotaxic device during experiments was attached to the skull surface with a light curing dental compound (Microglass® ,Kulzer, Germany). The experiments were conducted in an anechoic chamber lined with structured foam to reduce acoustical interferences from the environment and to minimize the reflections of call signals. The animals were placed in a sandwich-like holder which prevented gross body movements and the head was

immobilized by attaching the surgically affixed tube to a head-holder that allowed accurate repositioning (≤ 10 µm) of the animal in the stereotaxic device throughout experimental procedures.

The orientation of the skull and consequently the brain within the stereotaxic co-ordinates was determined by scanning the profile lines of the exposed skull in parasagittal and transverse directions at the first postoperative day. Details of the stereotaxic device, the procedures to determine the skull position and the reconstruction of the stimulation and iontophoresis sites are described elsewhere (Schuller et al., 1986). This method typically yields accuracy better than 100 µm in all three dimensions. As a control of the stereotaxic procedure, the stimulation and iontophoresis sites within the brain were marked by placing electrical lesions for post experimental anatomical verification and comparison with the stereotaxically known coordinates.

Electrical lesions were made by delivering -4 µA DC for 5 minutes through the tip of the stimulation electrodes. Starting from the stereotaxically placed lesion with its known coordinates all stimulation and iontophoresis sites could be calculated and compared with the three dimensional coordinates of the brain area under investigation to verify the range of stimulation.

The stimulation experiments within the PAG and PLA typically started on the third postoperative day and did not last longer than 5 hours per day. To insert the small stimulation electrodes and iontophoresis probes, a small hole was drilled into the skull using a miniature drill (custom made). Holes had a typical diameter of 200 µm and several probe penetrations with different rostro-caudal and medio-lateral inclinations were made through the same hole, in order to reach different locations. Coordinates of all penetrations, including lesioning penetrations, were referred to the reference point of the equipment, and thus could be mathematically transformed to the coordinate system of a standard series of frontal sections, especially prepared for this bat species as standard working brain atlas (T. Fenzl and A. Nixdorf, unpublished).

Electrical micro-stimulation

To localize the stereotaxic coordinates for the chronically implanted electrodes within the PAG (vocally active sites) and for the iontophoresis probes within the PLA (vocally active sites), electrical micro-stimulation was used.

For electrical micro-stimulation parylene-coated tungsten electrodes (type TM33A20, WPI Inc., Sarasota, USA) with impedances between 1.9 MΩ and 2.3 MΩ were used. The electrodes were placed on the brain surface and were lowered in steps of 100 µm or 200 µm using a piezoelectric micro-positioner. The exposed electrode tips of the metal electrodes had diameters of ~1 µm (sharpened tip). A sharpened tungsten wire mounted on an additional micromanipulator was inserted between the muscle fold and skull as indifferent electrode.

A Grass stimulator (type S 48, Grass, Quincy, USA) provided the electrical stimuli, which were delivered as current stimuli by a photoelectric stimulus isolation unit (type PSIU6, Grass, Quincy, USA) to the electrodes at negative polarity. Typical stimuli consisted of bursts of rectangular pulses of 0.1 ms width at a frequency of 1 kHz. The bursts lasted 15 ms and were presented at repetition rates of 6 bursts per second. Stimulus current ranged from 3 µA to 50 µA. 80 µA stimulus current was used only for wide range exploring within a particular area of interest.

The identical setup and stimulation paradigm was used with the chronically implanted electrodes except for the stimulus current, which was adjusted to the slightly changing threshold each experimental day (FIG. 2).

Iontophoresis within PLA

For reversible blockade of the PLA the glutamate antagonist kynurenic acid (Sigma-Aldrich, Steinheim, Germany) was used. Dissolved in 165 mM NaCl the concentration of the active agent was 75 mM (~ pH 9, adjusted with NaOH). Aliquots were kept frozen at –20 degrees centigrade. The iontophoresis probes were pulled on a capillary puller (model P-87, Sutter Instruments, Novato, USA) using borosilicate glass (type GB150F-10, Science Products, Hofheim, Germany). The tips of the probes typically had a diameter between 3 µm and 5 µm. Retaining current was 30 nA, the ejection current ranged between 200 nA and 250 nA, delivered by Neurophore BH-2 system (Medical Systems Corp., Greenvale, USA). The

iontophoresis probes were mounted either on a piezo drive or on a additional hydraulically driven micromanipulator (custom made), mounted on the x-axis of the stereotaxic device. Combined with electrical micro-stimulation in the PAG, a iontophoresis session typically lasted between 4 to 8 hours.

Sedation of the animal

To ensure a stable position of the animal during the whole period of session, Rompun® (Bayer, Leverkusen, Germany) with a concentration of 0.04% was applied subcutaneously (3.5 µl to 5.5 µl/min) through a winged needle infusion set (type Butterfly® -23, Abbott Laboratories, Dublin, Ireland) for sedation. The infusion set was attached to a syringe pump (TSE-Systems, Bad Homburg, Germany) driving a 2.5 ml micro-liter syringe (Hamilton, Switzerland). Forty minutes before the experiment started a initial dose of 0.4 ml sedative (0.04% Rompun®/0.9% NaCl) was injected subcutaneously.

Preparation and implantation of chronic stimulation electrodes into PAG

Single Teflon® isolated silver wires (type AGT0510, WPI, Sarasota, USA) with a diameter of 125 µm were stretched to straighten the wire. Fixed in the stretched position the Teflon® isolation was cut leaving a 2 cm bare silver ending on one side of the wire. According to the stimulation depth of the PAG sites the other end of the wire was cut leaving the isolation intact. This section ideally formed a sharpened bare silver tip (Ø 125 µm, length ~ 100 - 200 µm) for stimulation followed by an isolated segment long enough for the whole length of the penetration. Each single electrode was stereotaxically placed by holding it with a drill chuck (custom made) which itself was mounted to the piezo drive of the stereotaxic apparatus. After being placed in the PAG where electrical stimulation via these electrodes elicited the same vocalizations as electrical stimulation with the earlier described tungsten electrodes (see section electrical micro-stimulation), the silver wire electrodes were secured in place with cyanoacrylic glue to ensure safe positioning for further manipulations. After placing the electrodes a solid base using light curing dental compound cement was built up from the skull´s surface around the electrodes. The proper placement of the electrodes was immediately tested by the ability of the electrodes to trigger

vocalizations. A pair of IC-sockets (FIG. 1) was used as a connector box. Through each of the pins one of the bare silver wire endings was guided, cut to proper length and fixed around the outside walls of the socket. The IC-sockets together with the bare silver endings were mounted on the solid base with light curing dental compound cement. As a connector again IC-sockets with sharpened tips for easy fitting into the mounted sockets with its wires were used. Each single electrode could easily be controlled be switching the electrical stimulus between the socket pins using a remote controlled electric relay switch, interposed between the isolation unit and the stimulation electrodes.

Sound recording, processing and data analysis

The vocalizations of the bats were picked up with a ¼" ultrasonic microphone (type 4135, Bruel & Kjaer, Darmstadt, Germany), amplified and sampled (250 kHz) with an A/D converter board (type CIO-DAS16/M1, Computer boards Inc., Mansfield, USA), and stored on a personal computer. Signals were recorded with a custom-programmed recording program written in Agilent-VEE (version 6.0 pro, Agilent, USA). Vocalizations were spectrally analyzed and compared with known echolocation calls and communication calls of *P. discolor* (Fenzl & Schuller, 2002). For analysis of the recorded vocalizations the software "Bat Sound" (Petterson Electronic AB, Sweden) was used. Call length was evaluated using the envelopes of the vocalizations, peak amplitude of vocalizations was evaluated using the particular power spectrum and frequency shifts of vocalizations where measured within the spectograms.

A total blockade of PAG triggered vocalizations was reached when iontophoresis in the PLA led to an arbitrarily defined 75% failure to elicit vocalizations across the whole stimulation period for a time interval of at least 10 coherent minutes (refer to FIG. 2 for stimulation currents). One stimulation test period lasted in general 2 seconds with a total of 12 stimuli (6 Hz). Recovery was reached when during a stimulation period of at least 10 coherent minutes, 75% or more of the stimuli led to the emission of a vocalization. Refer to FIG. 3, 4 and 5 for temporal intervals between test stimulations.

Monitoring of animal reaction

During experiments the animals were continuously monitored by a TV camera (Teli, Tokyo, Japan) under infrared light illumination (LED array, 12 V/28 LED; Conrad Electronics, Germany) and observed on a monitor (TC-800 E4D, Osaka, Japan)

Tracer experiments

Wheat Germ Agglutinin marked with Horseradish Peroxidase (WGA-HRP, L 3892, Sigma, Germany) was iontophoretically injected into vocally active PAG sites, specified through electrical microstimulation in ten animals. Iontophoresis probes were pulled on a capillary puller (model P-87, Sutter Instruments, Novato, USA) using borosilicate glass (type GB150F-10, Science Products, Hofheim, Germany) and filled with a solution of approximately 2% in 0.9% NaCl. The tips of the electrodes typically had a diameter of 25 µm. The ejection current was 0.8 µA for 15 min, delivered by a custom made constant current source. Before the electrodes were removed, they were left on-site for additional 2 minutes after iontophoresis.

Neuroanatomical processing

After a survival period of 48 hours after WGA-HRP-injection, the animals were lethally anaesthetized with Nembutal® (16 mg/ml solution, 0.1 ml/10g BW) and trans-cardially perfused with 4 % glutaraldehyd in 0.05 M phosphate buffer solution. Cryoprotection for freeze cutting was done by soaking the brains with 30 % sucrose in 0.05 M phosphate buffer solution. The brains were embedded in egg yolk in a small Perspex chamber following a protocol (Schuller et al., 1986) that allows the alignment of the brain within the embedding block so that the ensuing section plane would optimally correspond to that of the reference brain sections of the brain atlas. The brains were freeze cut on a cryostat (Frigocut type 2700, Reichert-Jung, Germany) into 42 µm slices and generally three adjacent series were processed. Staining depended on the purpose of neuroanatomical processing and was done following different protocols: Nissl stain and fiber stain (Gallyas, 1979) to identify anatomical structures and a AChE procedure (Hardy et al., 1976) to identify cholinergic activity within the brainstem. Sections treated with TMB-tungstate

(Llewellyn-Smith et al., 1993) were counterstained with Nissl stain to visualize the transport of the tracer directly within anatomical structures.
Due to the standardized cutting procedure, data from individual brains could be well correlated to the sections of the reference brain, and thus made precise comparison of data from individual animals possible.

Animal care

Principals of laboratory animal care were followed and experiments were conducted under the regulations of the current version of German Law and Animal Protection. Reference Government of Bavaria (Az. Reg. v. Obb. 211-2531-37/98).

Results

Electrical stimulations within the periaqueductal gray (PAG), carried out via chronically implanted stimulation electrodes, triggers echolocation calls and communication calls, depending on the stimulation site. Kynurenic acid, applied iontophoretically into the paralemniscal area (PLA) seated contralaterally to the PAG stimulation site temporary blocks the production of echolocation calls derived from the PAG whereas ipsilateral blockade of the PLA only shows a weak influence on PAG induced echolocation calls. PAG induced communication calls could neither be blocked through ipsilateral nor contralateral blockade of the PLA. These differences display partly differentiated functional organizations of vocal controlling pathways for communication calls and echolocation calls below PAG level.

(1) Implants – Stability of chronical microstimulation

In each bat a pair of chronical electrodes for electrical microstimulation was implanted into vocally active sites within the PAG (FIG. 1a). The thresholds for eliciting identical vocalizations varied from probe to probe and from one experimental day to the next experimental day within relatively narrow limits. In FIG. 2a thresholds for four implants over a time period of 22 (animal 1) and 33 days (animal 2), respectively, are plotted. Only in experiment A the threshold declined by about 30%

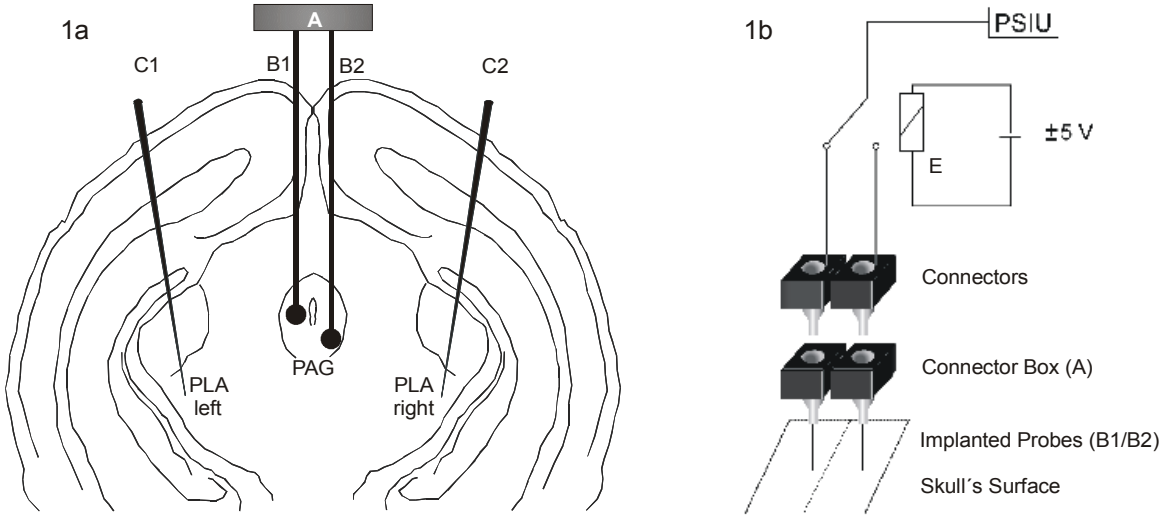


FIG. 1. Arrangement of chronically implanted stimulation electrodes and iontophoresis probes. (1a) The two chronically implanted silver wire electrodes (B1/B2) were connected to a connector box (A). They were positioned in order to stimulate different vocally active sites within the PAG. The iontophoresis probes (C1 and C2) allowed to pharmacologically manipulate regions of PLA. Stimulation sites in the PAG and iontophoresis sites in the PLA have been combined on an artificial level to clarify the arrangement of the probes. *In vivo* the iontophoresis probes and stimulation probes have a rostro-caudal offset of about 2000 µm. (1b) The connections to the stimulator output for the electrical stimulation electrodes are shown in detail including the remote controlled relay switch (E) and the photoelectric stimulus isolation unit (PSIU).

between the first measuring point at day 1 and day 33. The threshold in experiment B increases by 33%, in experiment C by 56% and in experiment D by 95%. The stimulation current in the PAG was individually chosen for each experiment at a supra-threshold level providing a one-to-one relationship between electrical stimulus and vocal answer. Figure 2b displays the mean percentage of stimulation currents above thresholds used with the chronical implants shown in Fig. 2a. For implant A the median value is 20% above threshold (P25=20, P75=25). The medians for implants B, C and D are 14% (P25=11, P75=15), 13% (P25=8, P75=14) and 14% (P25=7, P75=17) above threshold.

In animal 1 (FIG. 2a, graphs C and D) seven blockade experiments with comparable results were carried out. In animal 2 (graphs A and B) 12 blockade experiments were executed. The first nine experiments also showed comparable results until day 18 of the experiment. Two experiments at day 23 and day 29 allowed no PLA blockade of

PAG induced vocalizations although the iontophoresis probes where stereotaxically placed into identical PLA sites as in the experiments before. After a period of 22 days without subjecting the animal to further experiments blockade of PAG induced vocalizations was possible again at day 52 of the experiment (threshold for A: 60µA, threshold for B: 94 µA, data not shown).

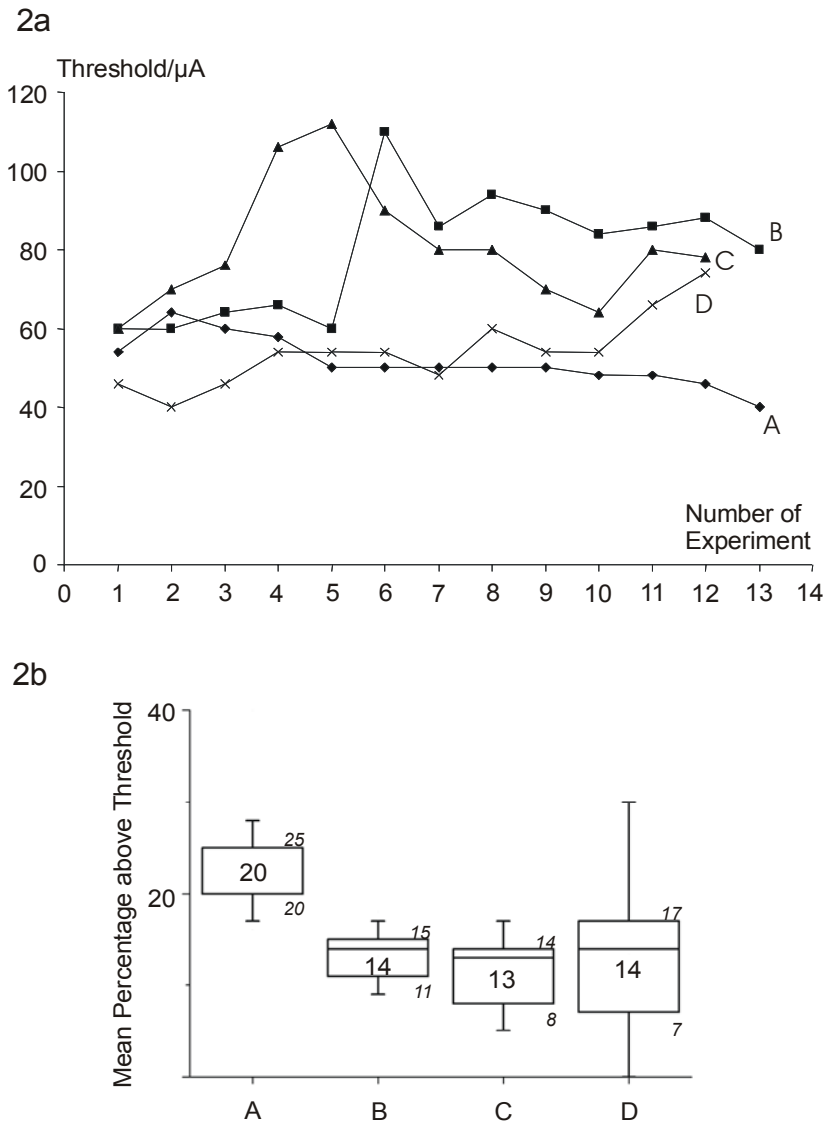


FIG. 2. Stability of stimulation threshold currents and time course at chronical microstimulation probes. Implantation of electrodes at day 0 of the graph 2a. (2a) Thresholds of four chronically implanted stimulation probes (A/B and C/D, in one animal each). Stimuli through implant A and B (animal 1) and through implant C (animal 2) elicited echolocation calls. Stimuli through implant D (animal 2) triggered communication calls. (2b) “Mean percentage above threshold”-values for all four implants (A to D) as plotted in 2a. Median values are indicated as numbers, additionally P25 and P75 values are plotted in italics. Note that the graphs in 2a display all experiments including experiments where blockade failed.

(2) Sedation of animals during experiments

To ensure that sedation had no influence on activation of electrically induced vocalizations, one experiment with PLA blockade (FIG. 3) and without blocking the PLA sites was carried out under identical depressant conditions. Neither the initial dose of 0.5 ml sedative prior to the experiment nor the continuous application of 6 µl/min showed any influence on the triggering of electrically induced vocalizations.
Again in all blockade experiments communication calls as well as echolocation calls could be elicited at the peak of sedation after injection of the initial dose without any deficits such as raise of threshold or disturbances in vocal patterns (see FIG. 4 and FIG. 5, initiation of individual experiments).

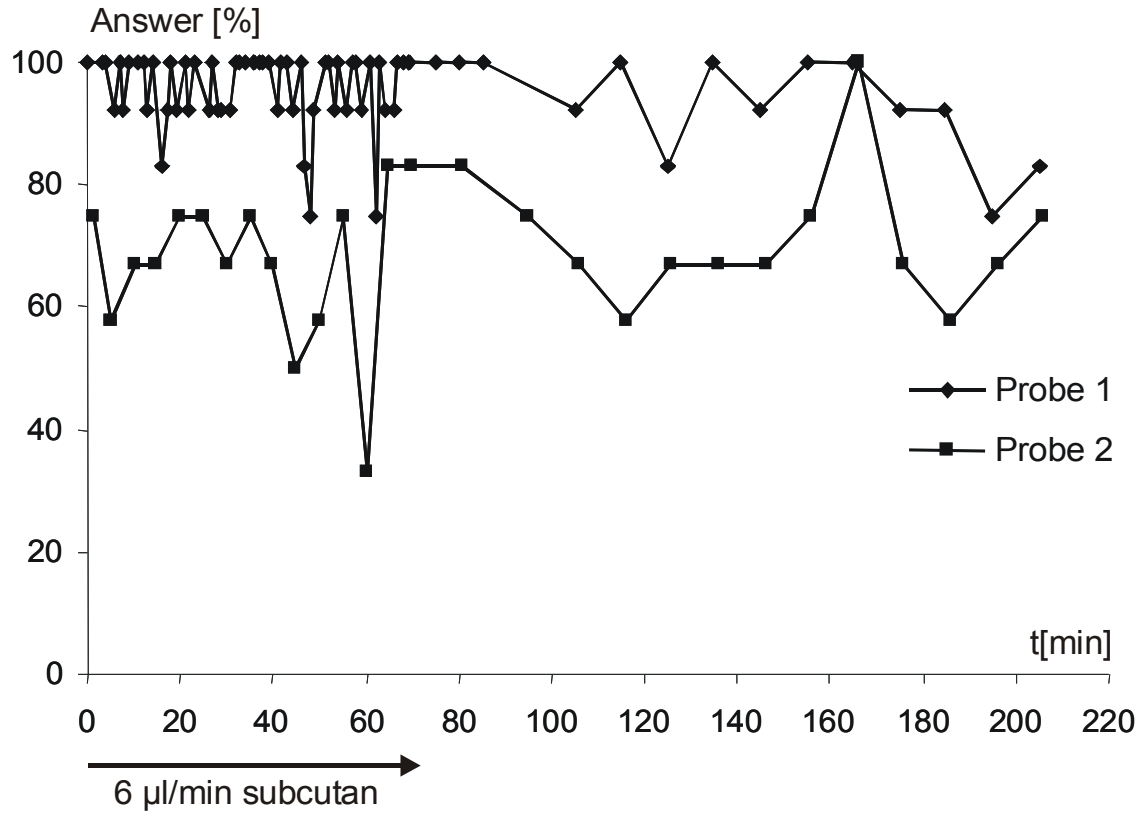
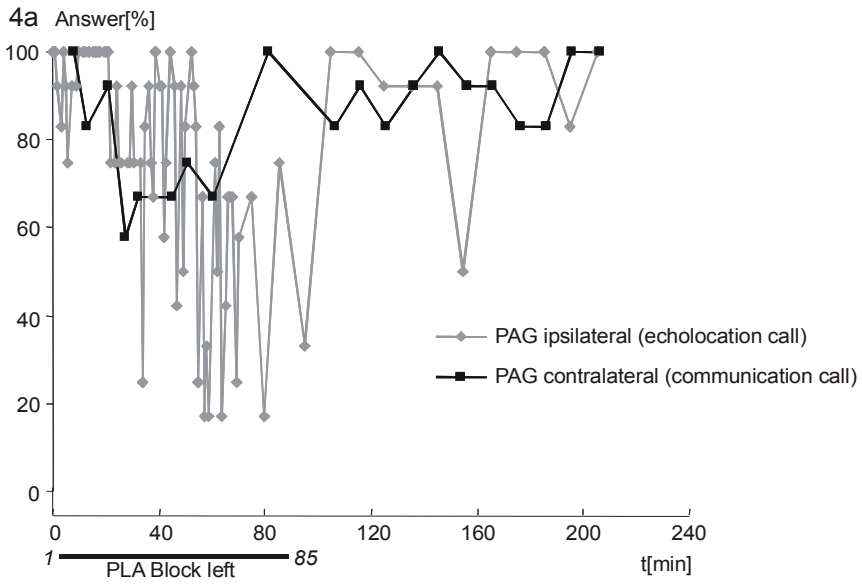
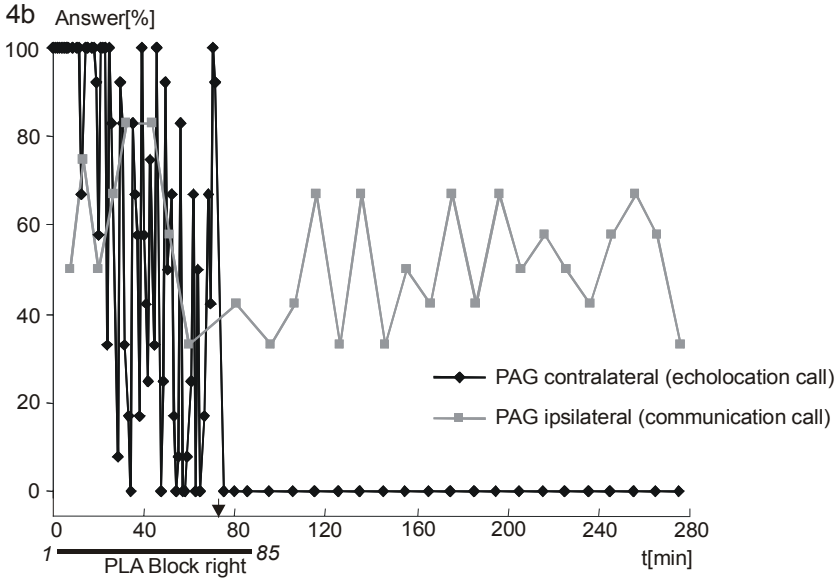


FIG. 3. Influence of sedation on vocal performances. Prior to electrical stimulation an initial dose of 0.5 ml sedative (0.04% Rompun® in 0.9% NaCl) was injected subcutaneously. Starting 30 min. later at minute 0 of the plot a continuous dose of 6 µl/min of 0.04% Rompun® was infused subcutaneously for a total of 205 minutes. The elicitability of electrically triggered vocalizations is shown for a time period of 205 minutes.

(3) Echolocation calls versus communication calls

Application of the broad spectrum and nonselective glutamate antagonist kynurenic acid into the PLA totally blocks PAG-induced echolocation calls whereas the production of communication calls is not affected at all. This effect is dependent on whether the PLA blockade is applied ipsilaterally or contralaterally to PAG stimulation sites. Figure 4 shows the time course of elicitability of vocalization for different combinations of ipsi- and contralateral PLA/PAG blockages/stimulations for echolocation calls and communication calls. In FIG. 4a kynurenic acid is applied to the left PLA of the animal for 85 min. Vocalizations, in this case echolocation calls, induced electrically within the ipsilateral PAG cannot be blocked although a depression can be seen between about 50 minutes after onset. The production of communication calls elicited within the contralateral PAG site is only slightly affected, rarely falling below 75% elicitability. A completely different pattern of vocal responses appears by PLA blockade contralaterally to the stimulated PAG inducing echolocation calls (FIG. 4b). Here kynurenic acid is applied to the right PLA of the animal for 85 min. The production of echolocation calls, elicited in the contralateral PAG site is totally blocked. While in the first 10 min of kynurenic application each electrical stimulus triggers a vocalization (answer = 100%), the response on PAG stimulation clearly starts to decrease after minute 10 indicating the beginning impact of the GLU antagonist. Shortly before minute 80 the production of contralaterally PAG induced echolocation calls is totally blocked persisting until the termination of the experiment after 240 minutes. In FIG. 4c kynurenic acid is applied bilaterally into the both PLA sites for 15 min. Again the production of PAG induced echolocation calls can be stopped totally developing shortly after the beginning of kynurenic acid application. This effect can be maintained throughout the whole experiment whereas the production of communication calls cannot be cut.


4a
Answer[%]
100
80
60
40
20
0
0
40
80
120
160
200
240
t[min]
1
85
PLA Block left
PAG ipsilateral (echolocation call)
PAG contralateral (communication call)


4b
Answer[%]
100
80
60
40
20
0
0
40
80
120
160
200
240
280
t[min]
1
85
PLA Block right
PAG contralateral (echolocation call)
PAG ipsilateral (communication call)

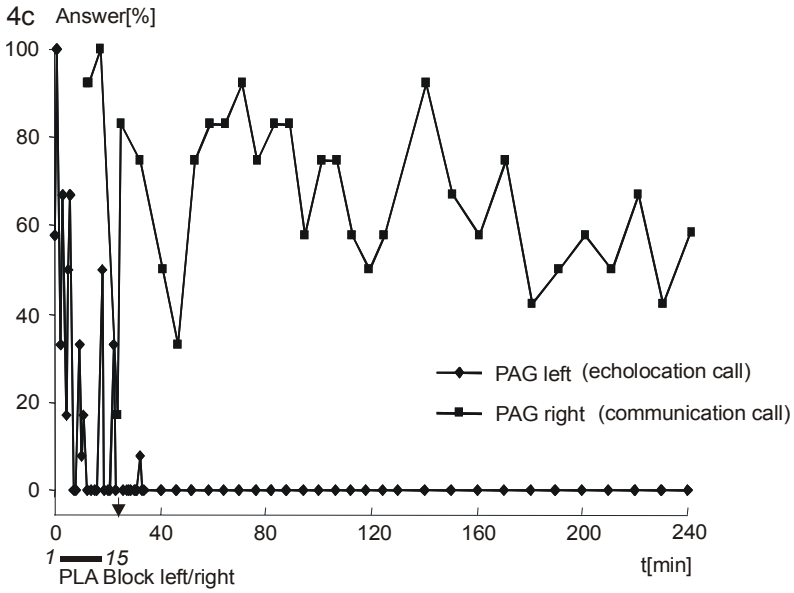
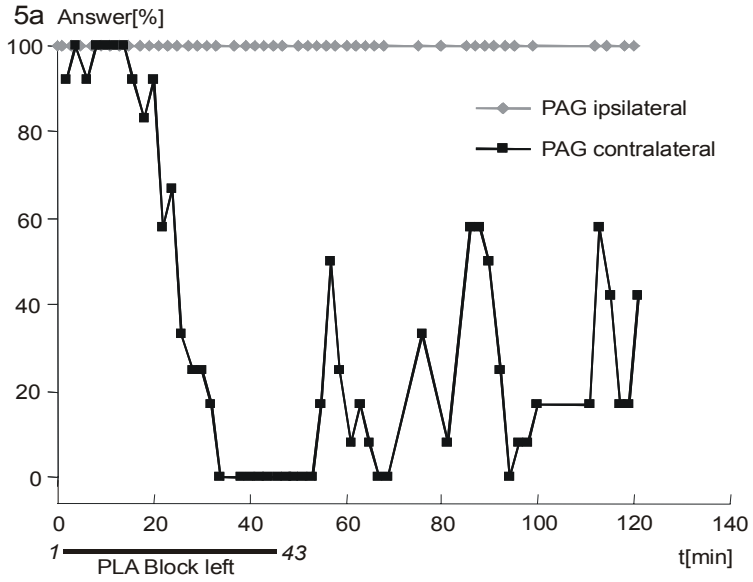
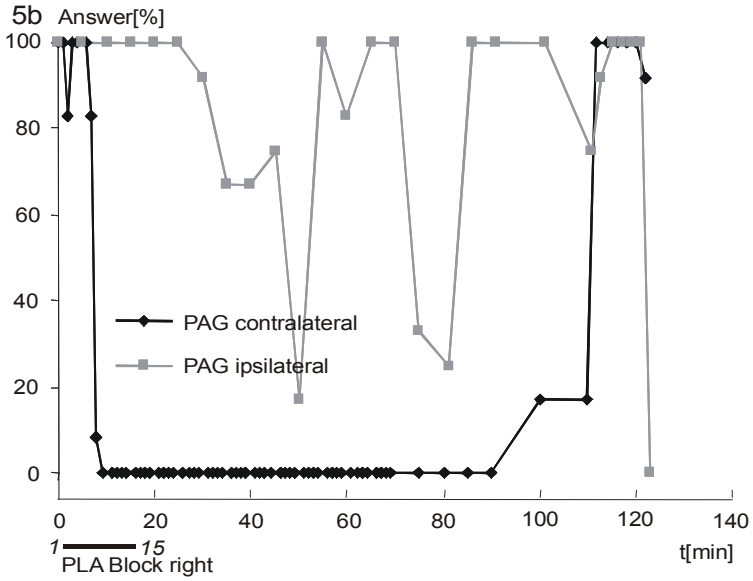

4c
Answer[%]
100
80
60
40
20
0
0
40
80
120
160
200
240
t[min]
1
15
PLA Block left/right
PAG left (echolocation call)
PAG right (communication call)

FIG. 4. PLA-located blockade of PAG-induced calls: The percentage of successful stimulations for eliciting vocalizations in the PAG is represented during and after kynurenic acid induced blockade of PLA. Echolocation calls are graphed with diamonds, the squares are used for communication calls. The kynurenic acid application is indicated by black horizontal bars. Onset and termination times of iontophoresis are given in italics. Stimulation success (%) was calculated for intervals of 2 seconds. Black arrows on the abscissa indicate the 25% blockade boundary.
(4a) Application of kynurenic acid to the left PLA. The ipsilateral production of PAG induced echolocation calls cannot be blocked although a slight depression can be noticed in the first half of the graph. The contralateral production of PAG induced communication calls is not influenced by the glutamate antagonist. (4b) A blockade of the right PLA totally blocks the production of contralaterally PAG induced echolocation calls while the ipsilateral production of communication calls again is not influenced. (4c) Bilateral blockade of both PLA sites again leads to a total depression of PAG induced echolocation calls while PAG induced communication calls can be recorded across the entire experimental run.

(4) Echolocation calls versus echolocation calls

Application of kynurenic acid into the PLA totally blocks contralaterally PAG induced echolocation calls whereas the production of echolocation calls from ipsilateral PAG sites is hardly affected. Figure 5 summarizes combinations of left or right PLA blockades and corresponding vocal responses of ipsi- or contralateral PAG induced echolocation calls. In FIG. 5a the GLU antagonist is applied to the left PLA for 43 min. Echolocation calls originated from contralateral PAG stimulation can be totally depressed for about 20 min before recovery starts after termination of kynurenic acid application. In contrast the ipsilateral PAG triggered echolocation calls are not affected at all. Figure 5b shows the same experimental approach with reversed PLA/PAG combination. Here the right PLA is temporarily blocked by kynurenic acid application for 15 min. Again only the contralaterally PAG induced echolocation calls are blocked totally for about 80 min before recovery starts. Although the ipsilaterally PAG induced echolocation calls show a decline in absolute answers to the electrical stimuli, they cannot be blocked totally. The experiment shown in FIG. 5c is identical to the experiment plotted in 5a except that kynurenic acid was applied for a second time into the identical PLA site. Here contralaterally PAG induced echolocation calls can be blocked while ipsilaterally triggered echolocation calls are hardly effected when kynurenic acid is applied for the first time. However, there is no supressing effect after a second contralateral application of the antagonist.


5a
Answer[%]
100
80
60
40
20
0
PAG ipsilateral
PAG contralateral
0
20
40
60
80
100
120
140
1
43
PLA Block left
t[min]


5b
Answer[%]
100
80
60
40
20
0
PAG contralateral
PAG ipsilateral
0
20
40
60
80
100
120
140
1
15
PLA Block right
t[min]

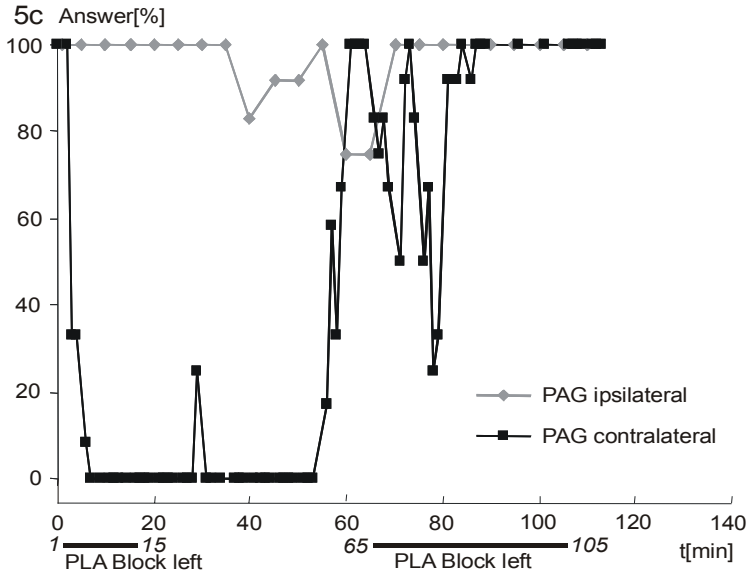
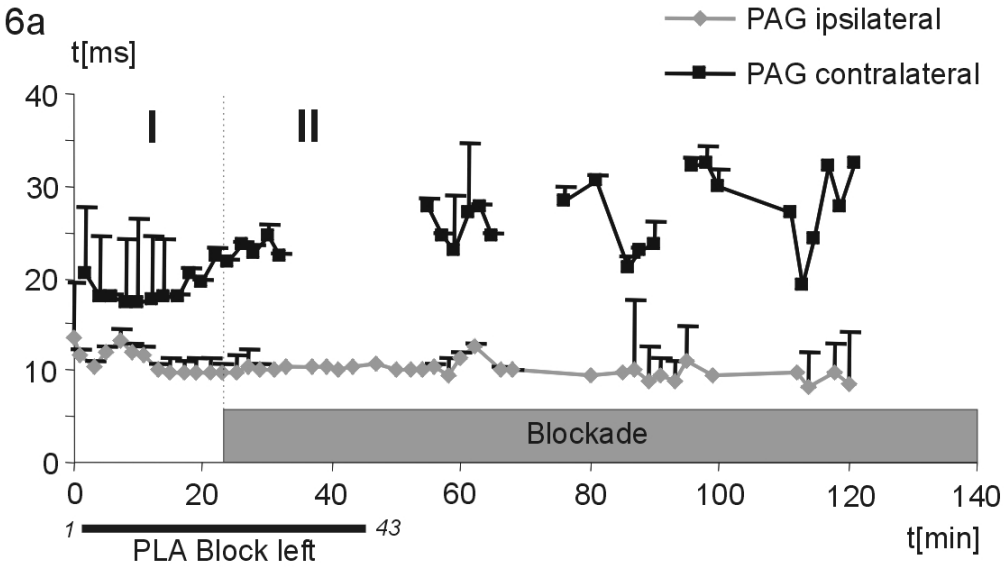

5c
Answer[%]
100
80
60
40
20
0
PAG ipsilateral
PAG contralateral
0
20
40
60
80
100
120
140
1
15
PLA Block left
65
105
PLA Block left
t[min]

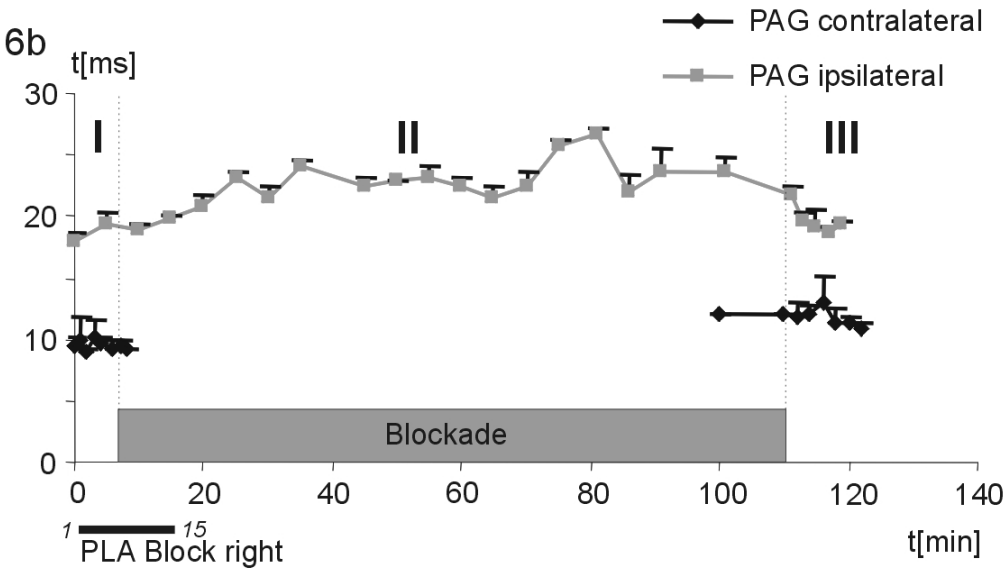
FIG. 5. PLA-located blockade of PAG-induced echolocation calls: ipsi- and contralateral differences. Refer to FIG. 4 for explanations on graph. (5a) Application of kynurenic acid to the left PLA does not influence the elicitability of ipsilaterally PAG-induced echolocation calls whereas it lowers dramatically the efficiency of contralaterally induced echolocation calls. The elicitability does not recover to the 75% mark within 120 min of experiment duration. (5b) A blockade of the right PLA totally blocks the production of contralaterally PAG induced echolocation calls while ipsilateral induced echolocation calls are little affected although some drops in elicitablity can be detected. (5c) Repeated application of kynurenic acid principally shows comparable results although at the second application the contralateral PAG induced production of echolocation calls cannot be cut off as effectively as shown for the first application.

Although the application time was prolonged from 15 min in the first cycle of the experiment to 40 min in the second cycle a total blockade cannot be accomplished anymore. At the beginning of the second application at 65 minutes a short trend of declining elicitability in PAG induced echolocation calls can be noticed, but the 25% criteria for a blockade cannot be reached. Already within kynurenic acid application time the contralaterally induced echolocation calls reach 100% elicitability again.

(5) Effect of PLA blockades on latencies of electrically induced PAG-vocalizations

Figure 6 shows that contralateral PLA blockades not only effect the elicitability of vocalizations within the PAG but also influence the latencies of vocal responses to electrical microstimulation. The blockade is marked with a gray bar at the bottom of each plot. Section one (I) represents the first part of the experiment until a 25% blockade was achieved, section two (II) represents the part of the experiment where blockade was active and vocal responses did not reach the 75% recovery boundary and section three (III) finally marks the part of the experiment where vocal responses recovered and crossed the 75% boundary again. Under the influence of kynurenic acid the mean latencies for vocal responses show significant differences when particular sections of the graphs are compared. Table 1 summarizes the statistical analysis for the latencies of ipsilaterally PAG induced vocalizations (echolocation calls as well as communication calls). The PLA blockade not only stops contralateral PAG-induced echolocation calls but also seem to have an influence on the latency of electrically induced echolocation calls triggered on ipsilateral PAG sites.


6a
t[ms]
PAG ipsilateral
PAG contralateral
I
II
Blockade
t[min]
1
43
PLA Block left


6b
t[ms]
PAG contralateral
PAG ipsilateral
I
II
III
Blockade
t[min]
1
15
PLA Block right

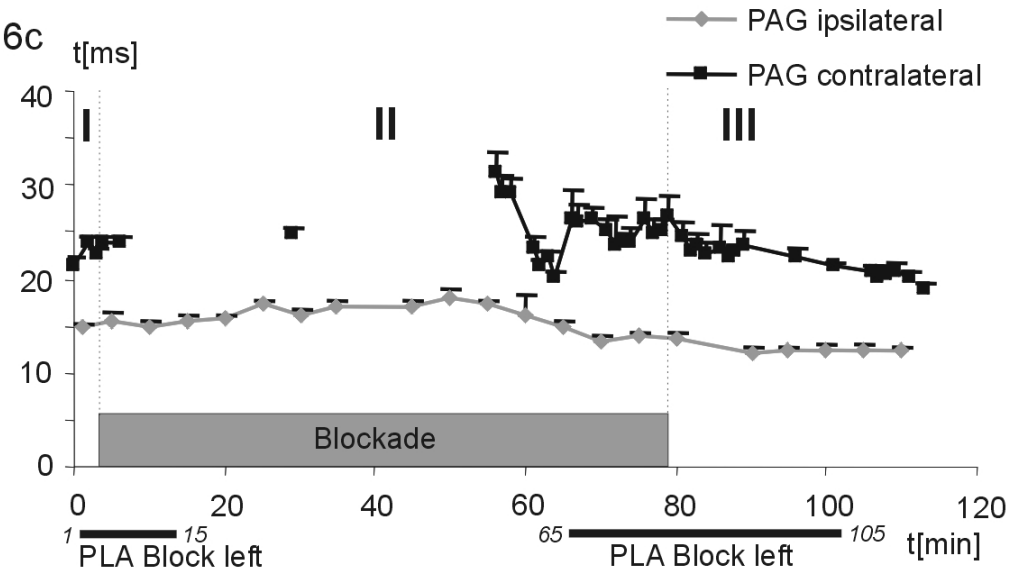

6c
t[ms]
PAG ipsilateral
PAG contralateral
I
II
III
Blockade
t[min]
1
15
PLA Block left
65
105
PLA Block left

FIG. 6. Mean latencies for vocalizations of experiments represented in FIG. 5. Figure 6a, b, c correspond to FIG. 5a, b, c, respectively. The black bars below each plot mark the period of kynurenic acid application. The gray bars on top of the abscissas indicate the duration of effective blockade (25% and 75% limits taken from FIG. 5). Gaps within the contralateral graphs mark 100% blockade. Standard deviations for mean latencies are plotted only positively. (6a) The blockade of contralaterally blocked echolocation calls (black graph) does not reach the 75% recovery before the termination of the experiment. The mean latencies of ipsilaterally (!) elicited calls show a significant difference ($p \leq 0.020$, table 1) when compared between the interval before or during contralateral blockade. (6b) The mean ipsilateral latency is calculated for 3 sections (I, II and III) before, during and after blocking the contralaterally elicited calls. The mean latencies differ for section I versus section II ($p \leq 0.025$, table 1) but not for section I versus section III ($p \leq 1.000$, table 1). (6c) The mean latencies for section II versus section III are significantly different ($p \leq 0.000$, table 1), whereas the data available before blockade are not sufficient.

(6) Histological verification of electrode positions

In *Phyllostomus discolor* the PAG is located around the mesencephalic aqueduct and has a lateral extension of about 1000 µm from the midline whereas the PLA (around 800 µm in its maximal medio-lateral dimension) is located medially, adjacent to the dorsal nucleus of the lateral lemniscus and contains neurons larger than those in surrounding areas (Fenzl & Schuller, 2002). Electrical lesions to mark the PLA stimulation and kynurenic acid-injection sites were placed stereotaxically 400 µm (range of lesion in dorso-ventral spread: 400 µm) ventral to the PLA (FIG. 7b) to ensure that the function of the PLA was not impaired during the ongoing experiments. Lesions in the PAG (7a) resulted from the microstimulation via the implants and could easily be detected in anatomical sections. In FIG. 7a the left lesion (L1) marks the site in the PAG where echolocation calls could be triggered while L2 shows the spot for the elicitation of a particular type of communication call.

(7) Tracer experiments

In ten animals WGA-HRP was iontophoretically ejected into sites within the PAG which had been identified as vocally active with electrical microstimulation prior to tracer application. In no case retrograde or anterograde connections between the

PAG and one or both PLA sites could be found. Strong anterograde and some retrograde projections however led from the PAG into several regions within the lower brainstem. Labelled fibers leave the ventro-lateral part of the PAG ipsilaterally to the injection site reaching the caudal part of the Medulla oblongata. At this level the labeled fibers separate into a ipsilateral and a contralateral stream. Although the ipsilateral stream is more distinct, both projections can be traced more caudally. Slightly dorso-lateral to the lateral nucleus of the formatio reticularis, the nucleus ambiguus can be identified (FIG. 7c/7d). At this and more caudal levels the anterograde projections from the PAG bilaterally terminate near the median half of the nucleus ambiguus and the medially adjacent part of the formatio reticularis, without forming clearly stained terminal endings.

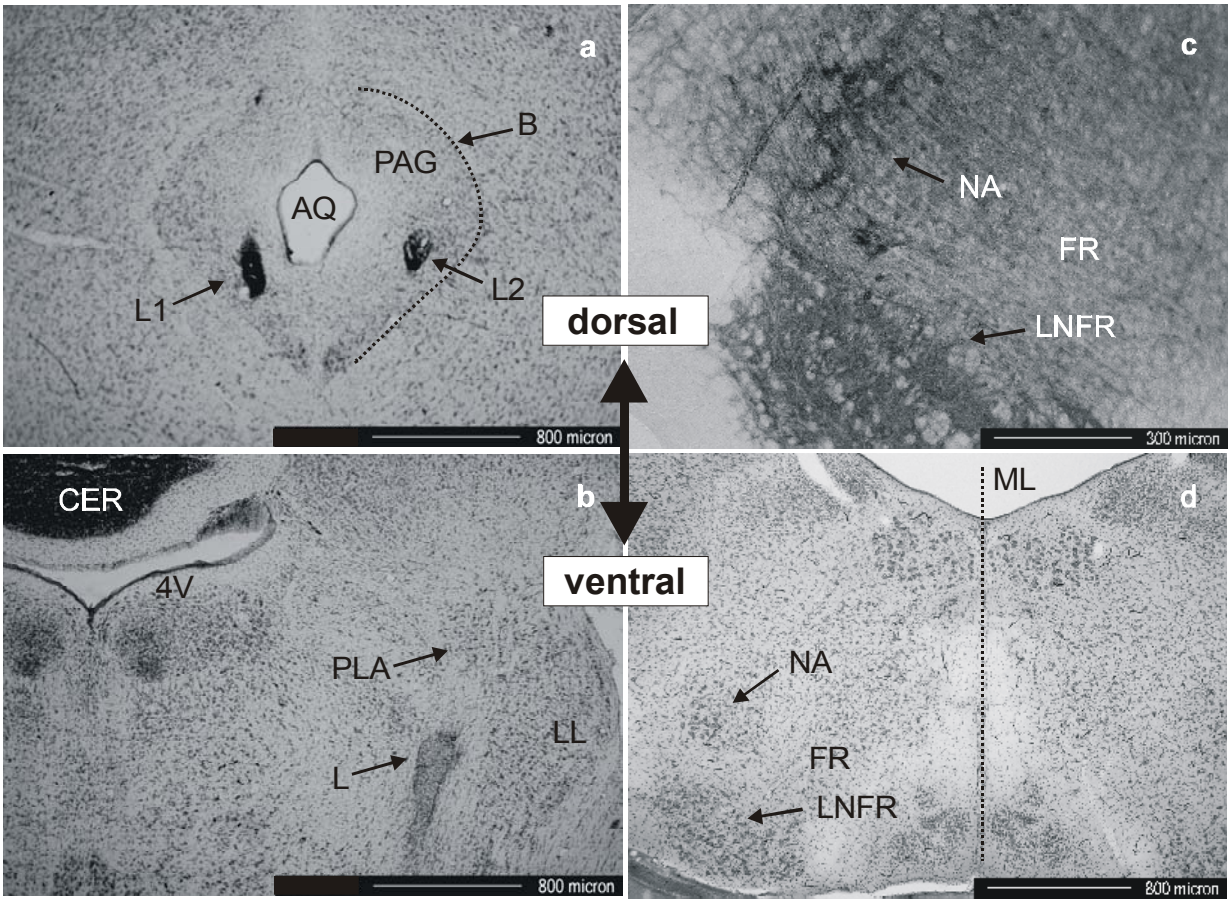


FIG. 7. Histological verification of electrode locations and location of the nucleus ambiguus. 7a, b and 7d show Nissl stained 42 µm frontal sections, 7c shows a AChE stain of a frontal 42 µm section. (7a) Two lesions (L1/L2) caused by repeated electrical stimulation through chronically implanted electrodes placed into vocally active sites within the PAG. AQ, aqueduct; B, boundary between PAG and surrounding tissue. (7b) Electrically induced lesion (L) 400 µm below the location of the iontophoresis probes in the PLA. Due to the 400 µm offset of the lesion below the PLA the function of the PLA during

further experiments was not influendced. CER, cerebellum; LL, lateral lemniscus; 4V, 4th ventricle. (7c) The AChE stained section represents the location of the cholinergic neurons in the nucleus ambiguus (NA) dorsolateral to the lateral nucleus of the formatio reticularis (LNFR) and lateral to the formatio reticularis (FR) itself. (7d) Overview of the lower brainstem at the level of the nucleus ambiguus; ML, dorsoventral median line. Section D corresponds to section C.

Figure	Section of graph	N	Mean latency	Standard deviation	Significance **ANOVA**	Comparison between plot section **Bonferroni**	Significance	Comparison between plot section **Mann-W.**	Significance
6a / 5a	I	12	11.16	1.37	-	-	-	I vs II	**0.020**
Echolocation call	II	32	9.99	0.82	-	-	-	-	-
	-	-	-	-	-	-	-	-	-
6b / 5b	I	2	18,67	1.12	**0.003**	I vs II	**0.025**	-	-
Echolocation call	II	17	22,61	1.96	-	I vs III	**1.000**	-	-
	III	5	19.71	1.23	-	-	-	-	-
6c / 5c	I	1	14.91	-	-	-	-	II vs III	**0.000**
Echolocation call	II	14	15.92	1.37	-	-	-	-	-
	III	6	12.61	0.58	-	-	-	-	-
4 a	I	22	40.51	3.77	-	-	-	-	-
Communication call	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-
4 b	I	8	47.66	3.99	-	-	-	I vs II	**0.019**
Communication call	II	16	52.52	4.19	-	-	-	-	-
	-	-	-	-	-	-	-	-	-
4 c	I	2	48.90	0.55	-	-	-	I vs II	**0.64**
Communication call	II	26	48.08	2.82	-	-	-	-	-
	-		-	-	-	-	-	-	-

Table 1: Statistics on latencies of PAG induced echolocation calls during PLA blockade experiments. “Figure” and “Section of graph” identify the data points used for statistics. “N” gives the number of data of mean latencies for each section of the graph in figure 6. “Mean latency” and “Standard deviation” refer to the data points of complete sections of a graph. ANOVA including a Post-Hoc Bonferroni test is applied on sections I, II and III of FIG. 6b. Mann-Whitney U-Test is applied on graphs with only two sections available for statistics (6a, 6c). Same criteria were applied on data for communication calls (FIG. 4), although not shown in FIG. 6.

Discussion

This study has demonstrated that the neural control of different types of calls involves different neural substrates for vocalization.

The periaqueductal gray is involved in the control of both echolocation calls and communication calls (Fenzl & Schuller, 2002), whereas the paralemniscal area, where only echolocation calls can be triggered (Fenzl & Schuller, 2002; Schuller et al., 1997; Schuller & Radtke-Schuller, 1990) is only involved the control of echolocation calls. The hypothesis that the production of particular communication calls and the production of echolocation calls must revert to differently organized neural substrates and pathways in the bat below the functional level of the PAG has been supported by the results presented in this paper. Thus the production of echolocation calls triggered in the PAG can totally be inhibited by a reversible blockade of the PLA, whereas, the production of communication calls elicited within the PAG is not influenced by such a blockade.

A straight forward vocal pathway from the PAG to the nucleus ambiguous/retroambiguus as described in the literature is not sufficient to explain these findings. A more complex organization of the descending vocal control system below the PAG level seems to be mandatory.

(1) Sedation of animals and stability of chronic implants

To ensure a stable position of the animal during the iontophoresis experiments Rompun® was chronically applied subcutaneously during the sessions. The reaction of the animal on external acoustic stimuli was used to evaluate the depth of sedation, meaning the animal responded with ear movement but without arousal or ample body movements. This condition could easily be maintained during the experiment by applying the permanent infusion of the sedative. The experiments typically started when the initial dose of Rompun® had its peak effect. At this time all bats showed perfect elicitability of vocalization (FIG. 3, FIG. 4 and FIG. 5) so that the effect of sedation on the ongoing experiments can be considered as minor. This sedative was adequate to both achieve optimal stability of the animal and minimal influence on vocal responses.

The stability of the chronically implanted electrodes for electrical micro-stimulation was very satisfactory, in that, both echolocation calls and communication calls without any change in spectral composition of the calls were elicited consistently over a long stimulation period of up to 33 days. The slight increase of stimulation thresholds for the individual electrodes (FIG. 2) can be attributed to an accumulation of glia cells and debris caused by the presence of implants. A decrease in elicitibility and/or quality of triggered vocalizations was not observed throughout the experiments. Also the intensity of motor reactions associated with vocalizations, like pinna and nose leaf movement with echolocation calls or mouth movements with communication calls, did not change during the experiments.

(2) Definition of PAG and PLA

Vocalizations are complex motor patterns imbedded into differentiated behaviors of an animal. It is well established that the periaqueductal gray (PAG) plays an important role in vocal control of communication calls (Jürgens, 1998; Jürgens, 2002), i.e., social calls can be triggered in several mammalian (Fenzl & Schuller, 2002; Kirzinger & Jürgens, 1991; Zhang et al., 1994) and non-mammalian species (Kennedy, 1975). In addition, echolocation calls can also be elicited in bats within restricted areas of the PAG (Valentine et al., 2002) separated from those areas where communication calls can be triggered (Fenzl & Schuller, 2002). The PAG seems to have control on both types of calls (Fenzl & Schuller, 2002).

Natural echolocation calls cannot be elicited in the PAG only. Very short latencies for eliciting ultrasonic vocalization and/or pinna movements were found in a restricted paralemnisal tegmental region rostral and medial to the dorsal nucleus of the lateral lemniscus (Schuller et al., 1997; Schuller & Radtke-Schuller, 1990).

Other examinations on functional aspects of the paralemniscal tegmental area are very rare. Covey (1993) differentiates between a dorsal paralemniscal zone and a paralemniscal zone on the basis of neural responses to acoustic stimuli. The paralemniscal area described by this author at the level of the intermediate nucleus of the lateral lemniscus and dorso-lateral to the medial superior olive is located more caudally than the paralemniscal area we define.

In contrast to a pure neuroanatomical definition, the paralemniscal area is functionally identified with electrical microstimulation, which offers a highly reliable possibility to

identify the vocally active area of the paralemniscal region. Histological verifications demonstrate the reliability of the functional identification. Only within a very restricted paralemniscal area vocalizations can be elicited at very low thresholds (3.4 µA – 15 µA) without, or with only minor pinna movements (Fenzl & Schuller, 2002; Schuller & Radtke-Schuller 1990).
Henkel and Edwards (Henkel, 1981; Henkel & Edwards, 1978) defined a tegmental area in the cat as the “paralemniscal zone” (PL) that corresponds partly in its connectional pattern to the situation found in the moustached bat (Schuller et al., 1997). However the area defined by the authors in the cat cannot be aligned properly with our anatomical data due to a lack of comparable anatomical cues.

In oculomotor function research a similar tegmental region is termed lateral tegmental region (LTR) where neurons, probed in the cat do respond to visual, vestibular and auditory stimulation (Gerlach & Their, 1995). According to these authors the PL described by Henkel and Edwards constitutes only a subdivision of the larger LTR. The comparable vocally active region in bats is even more restricted and only occupies the dorsal half of the paralemniscal zone as defined by Henkel and Edwards (Schuller et al., 1997).
Another characterization of the “lateral tegmentum underneath the inferior colliculus” is given by Jürgens (1994). This region along the medial edge of the lateral lemniscus is linked by the author with a vocal pathway from the PAG towards the periambigual reticular formation although only lesion-experiments were carried out leaving unanswered whether this region represents only fibers of passage or neurons involved in vocal control.
Due to the functional differences demonstrated by different authors, together with the definition of the tegmental region by various different methods, there is no clear definition of this area given, neither anatomically nor physiologically. It cannot be decided whether the different regions are part of a functionally coherent tegmental area responsible for diverse tasks or whether the tegmental area is an ensemble of independent functional regions.

The definition of the PLA used in this paper is strictly based on electrical microstimulation and the delimitation towards adjacent regions is recognized in a significant threshold increase for eliciting natural echolocation calls. Previous findings

about the tegmental region in bats (Fenzl & Schuller, 2002; Schuller & Radtke-Schuller, 1988; Schuller et al., 1997; Schuller & Pillat, 1998; Schuller & Radtke-Schuller, 1990) and this paper are all based on this functional definition and results can be directly compared.

(3) Differentiated vocal pathways ?

Could different types of vocalizations be processed via at least partly separated vocal pathways in the bat? The initial position of this question came from results that communication calls (Fenzl & Schuller, 2002; Suga et al., 1973; Valentine et al., 2002) and echolocation calls (Fenzl & Schuller, 2002, Suga et al., 1973; Suga & Yajima, 1988) could be elicited within the PAG, while within the PLA only echolocation calls could be triggered (Fenzl & Schuller, 2002; Schuller et al., 1997; Schuller & Radtke-Schuller, 1990). The two mesencephalic brain areas, PAG and PLA have never been investigated so far in bats in order to explore interactions between both regions in terms of vocalization. Our experiments clearly show a functional linkage between the PAG and both PLA sites for the control of vocalizations, which however reveals functional differentiation between two classes of vocalizations, i.e. communication calls and echolocation calls.

A vocal pathway for the production of communication calls as reported by several authors (Ennis et al., 1997; Vanderhorst et al., 2000; Zhang et al., 1995; Zhang et al., 1994) does not necessarily involve activity of the PLA. In this pathway a direct connection from the PAG towards the nucleus retroambiguus (NRA) is described. The NRA includes a group of premotor neurons which send direct projections to thoracic and upper lumbar motoneurons (Vanderhorst et al., 2000; Vanderhorst et al., 2000) involved in expiration, and to the nucleus ambiguus containing laryngeal and pharyngeal motoneurons (Holstege, 1989; Vanderhorst et al., 2000; Vanderhorst et al., 2000). This pathway alone cannot explain our findings that PAG induced echolocation calls can be blocked by PLA blockades but would be sufficient to explain that the production of communication calls could never be cut off totally by PLA blockade.

Jürgens (2000) demonstrated that injections of kynurenic acid into the ventrolateral pons around the ventral nucleus of the lateral lemniscus and superior olive of

monkeys, a region located ventral and caudal to the paralemniscal area and certainly not identical with the PLA in bats, can block periaqueductally elicited vocalizations . Only one type of communication call with characteristic frequency modulations over several kHz was effected by this blockade. Jürgens (2000) suggests that vocal patterns are generally controlled in different brainstem regions, and that vocalizations with frequency modulations seem to depend on an intact periolivary region. Echolocation calls of *P. discolor* are typically frequency-modulated calls (FM) covering a frequency range between 45 and 100 kHz with the 3^{rd} – 5^{th} harmonic (Rother & Schmidt, 1982). These FM calls can successfully be blocked in all experiments we carried out so far which could be analogous to what Jürgens found in his experiments with squirrel monkeys, however, in a different region.

The effect of PLA induced blockades on PAG triggered vocalizations strictly depends on the side of the application since echolocation calls can only be blocked when kynurenic acid is applied contralateral to the stimulation site in the PAG (FIG. 5). This is in contrast to what Jürgens (2000) describes for squirrel monkeys. He mentions that periaqueductally elicited vocalizations are only affected by ipsilateral, not by contralateral injection of kynurenic acid. This elementary difference could be due to different brain regions injected with kynurenic acid. The blockade sites in the monkey were all located in the ventrolateral pons, as described earlier and is definitely not the same area as the paralemniscal area, defined in this paper.

The results from the monkey cannot support the findings from Holstege (1989) and Zhang (1995) that the vocal control pathway consists only of a direct connection from the PAG to the nucleus retroambiguus in the medulla oblongata (Jürgens, 2000). Our findings do not rule out the direct connection from the PAG to the nucleus retroambiguus (see discussion on anatomical studies) but also demonstrate that a differentiated control of certain types of vocalizations via parallel or at least partly separated pathways for echolocation calls must exist. This evidence of a more complicated network for vocal control at the level below the PAG in the monkey as well as in the bat, underlines the broader significance of this concept on a mammalian level. The bat vocal control system cannot be considered as being specialized but rather being a general mammalian vocalization system with distinct emphasized features.

That only contralateral PLA blockades affect PAG induced echolocation calls while ipsilateral manipulations show no consequences is surprising. Besides strong reciprocal connections between the PLAs of both sides there are no major midline crossing projections that could account for the contralateral influence.

(2) Influence of PLA blockades on PAG induced vocalizations

It is noteworthy that the onset of a contralateral PLA blockade is extraordinary variable between only three minutes and almost 80 minutes under comparable experimental conditions. Slightly different positions of the iontophoresis probe at the PLA site may be responsible as even a deviation as small as 100 µm corresponds to almost 15% of the medio-lateral dimension (≈ 800 µm, Fenzl & Schuller, 2002) of the PLA in *P. discolor.* At marginal application sites kynurenic acid should take longer to influence a necessary number of neurons in the PLA, than when injected to the geometrically optimal PLA location. The onset time differences indicate that the suppressive effect of PLA inactivation depends on the size of population of PLA neurons involved. PLA blockades also leads to an increase of latency of PAG-triggered vocalizations indicating again influence of the PLA on cell elicitability.
The general role of the PLA is still not clear. Based on our findings the PLA should be a necessary link in the pathway for the control of PAG-induced echolocation calls, although the production of spontaneously emitted echolocation calls evidently survives bilateral electrolytic lesioning of the PLA (Pillat & Schuller, 1998).

Neuroanatomy

For the cat (Gerlach & Their, 1995) retrograde labelling in the ipsilateral PAG was a consistent result of tracer injections into the LTR (lateral tegmental region) but it cannot be certified that LTR and PLA are homologous brain regions.
In this study we could not demonstrate any direct connections between vocally active PAG sites and the PLA, using WGA-HRP injections into the PAG. Such connections could have been expected from the blockade experiments in the PLA and from findings that injections of WGA-HRP into vocally active sites within the PLA lead to

labelled neurons in the PAG (Schuller et al., 1997). The lack of labelling in *Phyllostomus discolor* may be a species-specific methodological problem with WGA-HRP. Quality of labelling in *Pyllostomus disclor* did generally not reach that in *Pteronotus p. parnellii* or *Rhinolophus rouxi* under identical conditions of tracer application.

Several authors report a direct projection from the PAG to the nucleus retroambiguus (Davis et al., 1996; Gerrits & Holstege, 1996; Holstege et al., 1997; Jürgens, 2002; Zhang et al., 1995; Zhang et al., 1994). The nucleus retroambiguus (NRA) itself is a group of premotor neurons in the caudal medulla sending direct projections to thoracic and upper lumbar motoneurons involved in expiration and to the nucleus ambiguus (NA) containing motoneurons to the larynx, pharynx and soft palate (Vanderhorst et al., 2000). Physiological studies revealed that the NRA is involved in vocalization activating expiratory and laryngeal muscles (Larson et al., 1994; Zhang et al., 1995). Ennis and coworkers (1997) demonstrated that periaqueductal gray neurons terminate in close contiguity to cholinergic neurons in the “compact, semicompact, loose and external formations of nucleus ambiguus”.

The NA in *Phyllostomus discolor* was identified using its cholonergic properties and is situated slightly dorsolateral to the lateral nucleus to the formatio reticularis. Massive ipsilateral projections from the PAG into the ventral brainstem target the caudal part of this identified NA-region. The labelled fibers ended in this region but terminals were not found. At medullary level projections from the PAG cross the midline and reach the corresponding regions. The fibers from the PAG to NRA in *Phyllostomus discolor* seem to serve also for vocal control, at least for a subgroup of communication calls.

Other control pathways for the control of vocalization besides the PAG to NRA route must exist as the blockade of PAG-elicited calls by inactivation of the PLA shows. However, the projections of the PAG into the region of the PLA as described by other authors either are very faint in *Phyllostomus discolor* or the PLA influences vocalization not via a serial but rather parallel route from the PAG to the common vocal pathway.

Summary and conclusion

Communication calls and echolocation calls can be elicited with electrical micro-stimulation through chronically implanted electrodes in the PAG. Direct projections from the PAG towards the region of the nucleus ambiguus/retroambiguus (NA/NRA) complex could be traced implicating a vocal pathway from the PAG to premotoneurons within this region. Reversible blockade of the vocally active PLA, where only echolocation calls can be triggered, totally block PAG induced echolocation calls but not communication calls. The PAG to NA/NRA pathway may not be the only pathway processing vocal activity. The PLA seems to be essential for the production of echolocation calls but not for particular types of communication calls elicited in the PAG. This suggests differential pathway organization for particular types of communication calls on the one hand and echolocation calls on the other hand. Whether the differentiation of pathways applies to the two classes of echolocation calls and communication calls in general, or whether it is more directly dependent on specific call properties in the acoustic pattern domain remains open to further experimentation.

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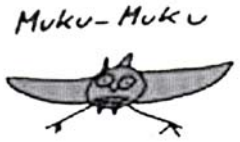

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