

## ABSTRACT

Title of Dissertation: NEURAL BASIS OF VIBRATION  
DETECTION IN LEPIDOSAURIAN  
REPTILES

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Biology

There are three potential pathways for detection of substrate vibration: cochlear, otolithic and somatosensory, reviewed in chapter one. How different lepidosaurian reptiles detect substrate vibration from these three pathways was explored from neuroanatomical and physiological perspectives. In chapter two, I described vibration sensitivity and the organization of the brainstem cochlear nuclei in the western snake (*Pantherophis obsoletus*). The western ratsnake is sensitive to low-frequency vibrations, comparable to other snakes. It has two first-order cochlear nuclei, nucleus magnocellularis (NM) and nucleus angularis (NA), similar to other reptiles. NM is small, while NA is relatively robust. In chapter three, I examined the connections and response properties of nucleus vestibularis ovalis (VeO) in the hindbrain of the tokay gecko (*Gekko gecko*). VeO receives input from the saccule, and connections of VeO mirror those of the cochlear nuclei, including an ascending projection to the central nucleus of the torus semicircularis. VeO neurons are sensitive to low-frequency vibration. In chapter four, I revisited a classic study to determine the connections and response properties of the snake torus

semicircularis. In the western ratsnake, the torus can be divided into a central nucleus and a paratorus, the latter receiving input from the spinal cord, nucleus myelencephali dorsalis in the spinomedullary junction, as well as auditory nuclei. Toral neurons are sensitive to low frequency vibration and have heterogenous response characteristics. In chapter five, I discuss future directions based on findings in my dissertation and highlight the importance of vibration detection for lepidosaurs.

NEURAL BASIS OF VIBRATION DETECTION IN LEPIDOSAURIAN  
REPTILES

by

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## Dedication

To snakes, for being so cool.

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## Chapter 1: Introduction

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Tympanic middle ears act as mechanical transformers that match impedance between air and the fluid in the inner ear and are important for hearing on land. When sound travels from a low-impedance medium like air to a much higher-impedance medium like water, or the fluid filled cavity of the cochlea, almost all of the acoustical energy is reflected. For animals with tympana, such as all birds and mammals, the tympanic membrane has low elastic resistance and is light weight, making it a good receiver of sound. Due to the difference in area between the tympanum and stapedial footplate, sound pressure levels are amplified up to 100-fold at the inner ear (Gummer et al., 1989; Hemilä et al., 1995; Rosowski et al., 1985).

Many species of lepidosaurs, a group comprising squamates (snakes and lizards) and *Sphenodon*, have secondarily lost their tympanic middle ears (Evans, 2016; Ford et al., 2021). Lepidosauria is the only living subgroup of the clade Lepidosauromorpha, sister to Archosauromorpha, which include modern groups Archosauria (birds and crocodilians) and Testudines (Chiari et al., 2012). Approximately 4500 species of lepidosaurs, out of a total of ~11000 (Uetz et al., 2021), are atympanate (lacking eardrums), including *Sphenodon*, snakes, chameleons, amphisbaenians and many others. The loss of the tympanic middle ear means

atympanate species are poorly suited to detect airborne sound pressure (Capshaw et al., 2022). They may, however, be able to detect mechanical waves present in dense media, such as the ground or plants, in the form of substrate vibration. Physiological studies on atympanate species show that the tympanic middle ear is not required for the detection of low-frequency substrate vibration (Capshaw et al., 2020; Christensen et al., 2012; Han & Carr, 2023; Hartline, 1971b; Womack et al., 2017). Vibrational stimuli from biotic, geophysical or anthropogenic sources are key components of the environment (*umwelt*) for lepidosaurs, including the 4500 species without tympanic middle ears.

The lack of tympana is also a trait associated with early tetrapod evolution. Early tetrapods had bulky stapes that are hypothesized to have served as a structural support in the skull, and their ability to hear airborne sound was likely poor. Earliest evidence of a tympanic middle ear is around the upper Carboniferous for primitive amphibians (Bolt & Lombard, 1985; Robinson et al., 2005; Sigurdson, 2008; Sigurdson & Bolt, 2010), and around early Triassic for different amniotes lineages (Clack et al., 2017), corresponding to approximately 70 million years and 100 million years, respectively, after vertebrates became terrestrial. During this period early tetrapods should have been primarily sensitive to substrate vibration rather than airborne sound. Exploring the underlying mechanisms for vibration detection in atympanate lepidosaurs allows glimpses into how early tetrapods and early amniotes process vibrational cues in their sensory world.

What sensory modalities underlie vibration detection? In John Tait's manuscript "Is all hearing cochlear?" written in 1932, he argued that the cochlea, otolithic endorgans such as the saccule, and "extra-labyrinthine" tremor receptors on the body could all sense vibrations in the surrounding medium. In my dissertation, I aim to answer questions regarding the neuroanatomical and physiological basis of vibration detection in lepidosaur species, from aspects involving 1) the cochlea, 2) the saccule and 3) somatosensory pathways. Specifically, I ask: what is the organization of cochlear nuclei in an atympanate species such as snakes? Do lepidosaurs use the saccule, an otolithic end-organ, to detect vibrations? How does the somatosensory midbrain contribute to vibration detection? The introduction will review each topic in order.

### *1. Vibration detection by the cochlea*

The cochlea can detect vibrations, either from direct stimuli impinging on the skull or induced by loud low-frequency sound. In tympanate vertebrates, the phenomenon is best understood in humans. Vibrations delivered to the mastoid or forehead of the skull create travelling waves on the basilar membrane comparable to stimulations from aerial sound, evidenced by cancellation of a vibration tone by sound (Stenfelt, 2007), generation of distortion-product otoacoustic emissions using vibrations (Purcell et al., 1998; Watanabe et al., 2008), and the similarity of vibration patterns on the basilar membrane between stimulations using sound and vibration (Stenfelt et al., 2003). This similar activation of the basilar membrane suggests that in tympanate species the neural pathways for detecting airborne sound are probably used

for sensing vibrations. On the other hand, how atympanate lepidosaurs process vibrations centrally is poorly understood.

One argument for divergence in the central processing of vibration between tympanate and atympanate species stems from the differences in their middle ears. In tympanate lizards, the tympanic membrane is anchored dorsally and rostrally to the curved part of the quadrate bone called tympanic crest, ventrally to the tympanic crest and the retroarticular process of the lower jaw, and caudally to soft tissue (Baird, 1970; Wever, 1978). The stapes or columella is a slender rod, and its distal, or posterolateral, end connects to the tympanic membrane via a cartilaginous extrastapes. The proximal, or anteromedial, end of the stapes expands to form a footplate on the oval window of the otic capsule. In atympanate species, a great degree of variation exists in the distal connections of the stapes. For example, in *Sphenodon*, the extrastapes is connected to a layer of dense connective tissue in the lateral occipital area of the head called the aponeurosis, which is further overlaid by the depressor mandibulae muscle and skin (Gans & Wever, 1976). In chameleons, the extrastapes expands into a plate and nests between the quadrate and the depressor mandibulae, with ligamentous connections to the quadrate, and in some species, the extrastapes has an anterior process that connects to the pterygoid plate (Olroyd, 2022; Wever, 1968). In some amphisbaenians, the stapes is extremely short, hardly projecting beyond the depth of the oval window, and a long cartilaginous extrastapes runs anteriorly through dense connective tissue and muscle overlying the low jaw (Gans & Wever, 1972).

Snakes are perhaps the most diverse and well-known group of atympanate lepidosaurs. The distal end of the stapes is largely made of cartilage and is held in contact with the quadrate only by a connective tissue capsule, allowing freedom of movement of the stapes. In some species of boids and uropeltids, there are additional cartilaginous disks between the stapes and quadrate; in *Typhlops pusillus*, the stapes is joined to the quadrate by a heavy ligament (Wever, 1978). These cartilaginous elements are thought to be homologous to extrastapedial structures in the lizard ear. The quadrate of the snake links the supratemporal to the articular portion of the mandible, and the linkage from mandible to otic capsule via the quadrate and stapes has been shown to bear importance for the detection of substrate vibration for snakes (Hartline, 1971b; Wever, 1978).

The proximal end of the stapes expands into a footplate and rests on the oval window of the otic capsule. The relative size of the stapedial shaft and footplate varies between species. Around the oval window and stapedial footplate, a crest of bones rises to form the crista circumfenestralis, a unique structure in modern serpents. This structure is further divided into three main components: crista prootica, crista tuberalis, and crista interfenestralis (Rieppel & Zaher, 2001). Variation in the height, degree of protrusion and amount of coverage occur among different species. Palci & Caldwell (2014) suggested that each element of crista circumfenestralis may have different developmental origins, and each component should be assessed individually. A thick periotic membrane anchors the stapedial shaft and the crista circumfenestralis.

In the snake inner ear, the cochlear duct is located ventral to the relatively large saccule, and its walls constrict to divide the duct into two distinct

compartments: the lagena and the limbus (Miller, 1968). The limbus is a round-oval to elongated-oval tube that lies directly ventral to the saccule, and supports the main auditory sensory organ, the basilar papilla. The stapedial footplate and fenestra ovalis lies close to the cochlea, and movement of the footplate generates perilymphatic pressure waves in the cochlear duct. Snakes lack the round window that most terrestrial vertebrates use to balance fluid movement from the oval window, but instead have a re-entrant fluid circuit. Displacement of perilymphatic fluid passes through scala vestibuli and scala tympani to excite hair cells on the basilar papilla, then returns to the stapes through the perilymphatic labyrinth. In the snake papilla, the hair cells are arranged in irregular rows, and are unidirectionally oriented with a tectorial membrane that overlies the full length (or almost the full length) of the basilar papilla (Miller, 1978; Miller & Beck, 1990; Wever, 1978). This contrasts with lizards, which have both unidirectional and bidirectional hair cells and varying degrees of tectorial membrane loss (Manley, 2002; Wever, 1978). In snakes, the basilar papilla varies greatly in length (0.1 mm to 1.5 mm) and hair cell number (50 to 1500) (Miller 1968; Wever 1978). With the exception of *Acrochordus javanicus* (which has around 1500 hair cells), the snake basilar papilla on average supports 245 hair cells. Although this number is greatly exceeded by that of mammals and birds (generally over 10000, see review in Gleich & Manley, 2000), it is comparable to iguanas and anguids, greater than chameleons and less than geckos and varanids. Among snakes, variation of hair cell number and basilar papilla length seem to correlate with ecological preference rather than phylogeny, with burrowing species

having the most robust basilar papilla, followed by terrestrial and then arboreal species (Baird, 1970; Miller, 1968; Wever, 1978).

The vestibulocochlear nerve (VIII) in snakes, like that of other reptiles, is composed of anterior and posterior divisions. The anterior division originates from the cristae of the anterior and lateral ampullae, as well as the utricular macula. The posterior division receives inputs from the saccular macula, the posterior ampulla, the basilar papilla and lagena macula, similar to their lizard relatives (Baird, 1970). The branch of the VIIIth nerve innervating the auditory papilla enters the brainstem, but prior to this study little was known about organization of central targets of the basilar papilla in snakes. In other reptiles, upon entering the brainstem, the auditory division of the VIIIth nerve bifurcates to terminate in two first-order cochlear nuclei, nucleus angularis (NA) and nucleus magnocellularis (NM). NM projects bilaterally to a second-order cochlear nucleus, nucleus laminaris (NL). Axons from NL and NA then project ventrally to the superior olive (SO), to the lemniscal nucleus and to the central nucleus of the torus semicircularis (homologous to the mammalian inferior colliculus). The lizard SO has a distinct dorsal component and ventral component (Tang et al., 2012; Foster and Hall, 1978).

In snakes, the cochlear portion of the VIIIth nerve targets dorsal nuclei in the rostral medulla, similar to projections in other reptiles (Carr & Boudreau, 1991; Han & Carr, 2023; Leake, 1974; Tang et al., 2012; K. L. Willis & Carr, 2017), although interpretation and description of its first order projections vary. Holmes (1902) described a single “small and ill-defined” nucleus dorsalis. Weston (1936) described a

single cochlear nucleus which was homologous to NA, while ten Donkelaar & Nieuwenhuys (1979) found only a “dorsal magnocellular nucleus”. Schwab (1979) confirmed a single cochlear nucleus but noted that in *Cylindrophis* it could be divided into anterior and posterior subnuclei. In *Python reticulatus*, Molenaar (1976) identified a small rostral NA and a large caudal NM. Miller (1980) also differentiated NM and NA, and further separated NM into nucleus magnocellular medialis and nucleus magnocellularis lateralis to align with the nuclear subdivisions of the caiman in Leake (1974) but noted that in some species NA is not “sharply demarcated” and the two magnocellular nuclei may be merged. These results were agreed upon by DeFina (1981) and Defina & Kennedy (1983).

Additionally, many researchers noted the lack of NL in snakes (Molenaar, 1976; ten Donkelaar & Nieuwenhuys, 1979; Weston, 1936). Yet, Miller (1980), followed by DeFina (1981), described the presence of a small layer of cells beneath NM, which he deemed NL, but observed that in some species the nucleus is indistinct. Fibers from the first order cochlear nucleus were observed to course ventrally to the SO (Holmes, 1902; ten Donkelaar & Nieuwenhuys, 1979) and these fibers progressed rostrally to form the lateral lemniscus and terminate in the caudal colliculus and nucleus isthmi (Molenaar, 1976); however, whether SO can be further subdivided, like in lizards (Foster & Hall, 1978; Tang et al., 2012) was not discussed.

The snake torus semicircularis consist of a large “paratorus” and a small central nucleus (Senn, 1969; Senn & Northcutt, 1973). There are no studies on the connections of the snake auditory midbrain. Studies in lizards show that the central

nucleus of torus semicircularis receives inputs from NL, NA, SO and the lateral lemniscal nuclei. The central nucleus then projects to nucleus medialis in the dorsal thalamus (Foster & Hall, 1978; Yan et al., 2010). Terminations from the nucleus medialis could be further traced to the medial dorsal ventricular ridge and the striatum (Bruce & Butler, 1984; Foster & Hall, 1978). Given the changes in their ears compared to lizards, determining whether or not the snake's central cochlear pathway follows the same general pattern requires further examination using modern tracing techniques.

Wever & Vernon (1960) were the first to show that the snake cochlea is capable of responding to vibrational cues. They used cochlear microphonic recordings to show that the quadrate bone, which contributes to the cranial kinesis that snakes are widely known for, is an essential component in these scenarios. Removal of the quadrate leads to notable declines in response thresholds. Further findings, detailed in Wever (1978), showed that to evoke cochlear potentials, the quadrate, to which the stapes was attached, was not required to move in a swaying motion about one of its joints. Instead, the articulations were loose enough for it to vibrate as a whole. Another important contribution from Wever & Vernon (1960) and Wever (1978) was their comparative approach; auditory sensitivity curves from 18 species of snakes across 6 families were obtained. In general, snakes showed highest sensitivity for sound frequency of 200-400 Hz, declining on towards the edges of the range. No differences were found based on phylogenetic background or habitat preference, although there was a significant correlation between hair cell number and the strength of the cochlear microphonic.

Hartline & Campbell (1969) evaluated how well snakes could respond to airborne sound and substrate vibration by performing extracellular recordings in the torus semicircularis. Rattlesnakes were sensitive to sound or vibration delivered to areas along the body, extending beyond midsection. This gave rise to the notion that these snakes could use both the inner ear and somatosensory input to sense vibration (see section 3 of this chapter for somatosensation). In a later experiment, Hartline (1971b) explored the responses of the torus to sound and vibration by addressing the inner ear and the spinal cord separately. Following transection of the spinal cord to isolate the contribution of the inner ear, they found that the auditory system of the rattlesnake was sensitive to low frequency vibrations around 150 Hz. They also noted that following destruction of the inner ear, stimuli delivered directly to the head or stapes failed to elicit a midbrain response, indicating that only the auditory system was activated when the vibration stimuli were delivered to the head. The ball python has a similar vibration sensitivity to low frequencies based on measurements of auditory brainstem response thresholds (Christensen et al., 2012). However, while both studies confirm contributions of the inner ear, and Wever (1978)'s microphonics experiments illustrate the importance of the cochlea, it remains unclear whether the auditory brainstem response recordings and auditory midbrain recordings include inputs from otolithic endorgans.

## 2. Vibration detection by the saccule, an otolithic endorgan

The saccule in vertebrates can be generalized as an inner ear organ containing dense calcareous otoliths or otoconia coupled to sensory hair cells. During

acceleration, the inertia of the heavier otoliths bends the sensory hair cells on the saccular macula, allowing the animal to detect motion of the surrounding media.

While the saccule serves as a vestibular endorgan for all jawed vertebrates (gnathostomes), it also has an auditory function in fish and amphibians (for reviews, see Ladich & Popper, 2004; Smotherman & Narins, 2004).

Sound has two components, pressure and particle motion. In an aquatic environment, the density of the fish and water is similar, and underwater sound causes motion of the body. This motion causes the otolith in the saccule, which is dense and weakly bound to surrounding tissue, to shear and bend hair cells, similar to the saccule's response to a linear acceleration (Schellart & Popper, 1992). The nerve fibers originating from the fish inner ear project to four octaval nuclei in the medulla, namely the anterior nucleus, nucleus magnocellularis (not to be confused with NM in reptiles), the descending nucleus, and the posterior nucleus (McCormick, 1999). All inner ear end-organs project to each of the four nuclei, but only the dorsomedial zones of the descending nucleus and, in some species, the anterior nucleus, receive input from the saccule and project to the torus semicircularis. The dorsomedial region of the descending nucleus sends bilateral projections to a more medially located second-order octaval nucleus (McCormick, 1999). Additionally, a commissural pathway connects the right and left dorsal descending nuclei, with some cells sending axons both to the torus semicircularis and the contralateral descending nucleus (Edds-Walton, 1998). Best frequencies of saccular fibers of the plainfin midshipman (*Porichthys notatus*) were distributed bimodally at 50 Hz and 100 Hz (Weeg et al.,

2002). Physiological recordings of neurons in the descending nucleus made in the toadfish (*Opsanus tau*) show best frequencies of 84-141 Hz, corresponding to that of saccular fibers, as well as directional sensitivity (Walton 2008).

While papillar endorgans (the basilar papilla and amphibian papilla) have appeared in amphibians to be better sensors to airborne sound pressure, the saccule retains its acoustic function as a detector of seismic vibrations (Lewis & Narins, 1999). The most dominant seismic vibrations relevant to animals dwelling on the surface of the ground are Rayleigh and Love waves (Narins, 1990). Due to the greater density of the saccular otolith compared to surrounding tissue, seismic vibrations cause relative movement of the otolith, which facilitates bending of underlying hair cells. Movement of particles in Rayleigh waves is elliptical and thus has both vertical and horizontal components, which effectively stimulates hair cells oriented in the same directions (vertical and horizontal) on the saccular macula (Hetherington et al., 1986). In anurans, the primary auditory nucleus is the dorsal medullary nucleus. The dorsomedial part of this nucleus receives input from the basilar papilla, the intermediate area receives input from the amphibian papilla, and the ventral neuropil receives input from the saccule. The dorsal medullary nucleus projects both to its contralateral homologue as well as to the torus semicircularis (Feng, 1986). A second-order octaval nucleus, the superior olivary nucleus, receives bilateral projections from the dorsal medullary nucleus, the lateral octaval nucleus and the caudal nucleus or medial vestibular nucleus (Horowitz et al., 2006). Recordings in the VIIIth nerve showed that saccular fibers respond to seismic vibrations and low frequency airborne

sound (Christensen-Dalsgaard & Narins, 1993; Koyama et al., 1982; Lewis et al., 1982). While there are extensive studies on the response properties of neurons in the dorsal medullary nucleus (e.g. Fuzessery & Feng, 1981; Hall & Feng, 1990; van Stokkum, 1987), the stimuli used were mostly airborne sound and below the detection threshold of saccular nerve fibers, and few studies have specifically characterized the response properties of the first-order saccular nuclei in the frog medulla. Christensen-Dalsgaard & Walkowiak (1999) investigated response properties of neurons in the saccular nucleus (the ventral neuropil of dorsal medullary nucleus) and the caudal nucleus and identified neurons with best frequencies of 40-70 Hz and 200-400 Hz. In non-anurans, the dorsal medullary nucleus is absent, and the saccule projects to a more dorsal area ventral to the intermediate area and a more ventral magnocellular portion of the ventral zone. The dorsal area has been considered homologous to the dorsal portion of the caudal nucleus and neuropil ventral to the dorsolateral nucleus in anurans. Both saccular areas in non-anurans appear to project to the midbrain (Will & Fritsch, 1988).

In amniotes, vibrational stimuli impeding on the skull of the animal should cause movement between otoconia and the underlying macula, comparable to amphibians. Otolithic end-organs are natural accelerometers and are intrinsically sensitive to low frequency vibrations (Grant & Curthoys, 2017). Saccular fibers in the squirrel monkey were sensitive to 200-400 Hz vibrational stimuli and loud (>100 dB) sounds (E. D. Young et al., 1977). In the cat, similar sensitivities were reported from recordings of irregular discharging saccular fibers originating in the saccule striola

(McCue & Guinan, 1994, 1995). In guinea pigs the ganglion cells that innervate hair cells in the striola of the saccule phase-locked to both acoustic and vibrational stimuli up to 1500 Hz and are sensitive to vibration frequencies up to 500 Hz (Curthoys et al., 2016).

However, so far there is little evidence to suggest that vibrations detected by the saccule are processed as auditory signals in amniotes. There is anatomical support for projections from the saccule to the central auditory system in mammals. The saccule projects to cochlear nuclei in the Mongolian gerbil, where sparsely labelled terminals were seen in the anteroventral cochlear nuclei, posteroventral cochlear nuclei, dorsal cochlear nuclei, and granule cells after injection of HRP into the saccular macula (Kevetter & Perachio, 1989). Another group reported en passant terminals in the “interstitial nucleus of stria of Held”, which comprise of medial, spindle shaped neurons located in the granular cell domain, following label injection into the posterior part of the saccule in the guinea pig (Burian et al., 1989, 1991; Burian & Gstoettner, 1988; Gstoettner et al., 1991). A few saccular fibers running lateral to the inferior cerebellar peduncle likely terminated in cochlear nuclei in the cat (Yingcharoen et al., 2003). Similarly located saccular fibers terminating in the ventral cochlear nucleus were seen in macaques (Newlands et al., 2003). However, multisensory integration has been shown to occur during early stages of auditory processing in mammals. Somatosensory input from the trigeminal ganglion, spinal trigeminal nucleus, dorsal root ganglia, and dorsal column nuclei terminate mainly in the granule cell domain but also in magnocellular regions of the ventral cochlear

nucleus and dorsal cochlear nucleus and are involved in the suppression of self-generated sounds and provide information about the orientation of the ear relative to the body (reviewed in Wu et al., 2015). Brainstem vestibular nuclei as well as projections from other vestibular endorgans in the inner ear have also been shown to send projections to the cochlear nuclei and are hypothesized to facilitate integration of head movements during sound processing (Barker et al., 2012; Bukowska, 2001; Wu & Shore, 2018). The early integration of multisensory information in auditory processing, and the lack of a dedicated relay nucleus for saccular input, confounds the argument for saccular hearing in mammals.

There is also little support for saccular hearing in birds. In the pigeon, tracer application to individual inner ear end-organs shows that the saccule only projects to vestibular nuclei, which do not terminate in the auditory midbrain (Dickman & Fang, 1996). It is worth noting that in the chicken, there is a nucleus located between nucleus laminaris and nucleus angularis, the *regio intermedius*, which sends projections to the rostromedial torus semicircularis (Y. Wang & Karten, 2010). The rostromedial torus has been reported to contain neurons that respond to low frequencies (Theurich et al., 1984). Physiological recordings in the ventral and medial margins of nucleus angularis, which in retrospect could be part of *regio intermedius*, also report neurons with best frequencies below 50 Hz (Warchol & Dallos, 1989).

The ascending pathways from the saccule in lepidosaurs (squamates, including snakes and lizards, and *Sphenodon*) have not been examined. No studies have specifically labelled the saccular macula and traced its central projections. In

lepidosaurs, the anterior branch of the VIIIth nerve innervates the anterior and horizontal semicircular canals and the utricle; the posterior branch innervates the saccule, lagena, basilar papilla, papilla neglecta, and the posterior canal (Mulroy & Oblak, 1985; Retzius, 1884). In lepidosaurs, many have noted a small but distinct nucleus in the dorsal octaval column that does not receive input from the basilar papilla. Described by Glatt (1975b) as “nucleus lagenaris”, it is a spherical nucleus that is located laterally in the dorsal part of the acoustic tubercle. Rostrally, it extends to the caudal end of nucleus laminaris and caudally, it extends slightly beyond the caudal end of nucleus magnocellularis. While this nucleus was first described in the caiman, in the caiman it is smaller in relative size compared to lizards and extend rostrally to the level of nucleus angularis (Glatt, 1975a, 1975b). Nucleus lagenaris was also described in turtles but its position is more ventral, and borders were indistinct (Glatt, 1975b). Moreover, the “lagenar” nature of fibers terminating in this nucleus was based on visually retracing its path back to the posterior ganglia in cresyl-violet and silver albumose stained sections. Thus, there was insufficient evidence to determine the endorgan of origin for this structure and to determine whether the three nuclei in lizards, caiman, and turtles share similar connections. The nucleus was mentioned in Foster & Hall (1978) as nucleus vestibularis ovalis in *Iguana iguana*, but not discussed in detail as the study focused on central auditory pathways. It is worth noting that removal of the VIIIth nerve ganglia led to degeneration of terminals in nucleus vestibularis ovalis, while smaller lesions in the intraotic ganglia did not. Barbas-Henry & Lohman (1988) used HRP to label the posterior root of the VIIIth nerve in *Varanus exanthematicus*, and found terminals in

this nucleus as well, naming it “caudal cluster”. Considering that this nucleus received projections from the posterior branch of the VIIIth nerve but possibly not from the intraotic ganglia, the sacculle remains a promising candidate for endorgan of origin. This nucleus was also depicted in Fig. 10 of Weston (1936) and Fig. 16 of ten Donkelaar & Nieuwenhuys (1979) but not discussed in the text. More recently, Song et al. (2013) confirmed the existence of nucleus vestibularis ovalis in *Gekko gecko*. However, contrary to earlier studies, Song et al. (2013) reported that this nucleus did not receive primary projections. Considering the sacculle macula can be small in Gekkota (see *Platydictylus* in Retzius, 1884), it is possible that the saccular branch of the VIIIth nerve was excluded during iontophoresis of tracer. Song et al. (2013) also described some retrogradely labelled cells in this nucleus following injections of neurobiotin in the oculomotor nucleus. Further neuroanatomical studies are needed to resolve its peripheral innervation and ascending circuitry.

There have been no saccular nerve recordings in lepidosaurs, or recordings of the VIIIth nerve using vibration as stimuli. There have been many physiological studies of the auditory nerve fibers using sound as stimuli (Christensen-Dalsgaard et al., 2021; Eatock & Manley, 1981; Holton & Weiss, 1983; Johnstone & Johnstone, 1969; Köppl et al., 1990; Manley, 1977; Manley et al., 1988; Turner, 1987; Weiss et al., 1974, 1976), where activation of saccular hair cells was unlikely considering the high sound amplitudes required to elicit sufficient head vibrations. Unsurprisingly, in the studies which also retrogradely labelled fibers to the inner ear, the authors did not note saccular contributions (Manley, 1977; Weiss et al., 1974, 1976). These findings

cannot eliminate the potential contributions of the saccular nerve and its central targets to detecting substrate vibration.

### 3. Vibration detection by somatosensory pathways

In mammals, vibrations from the head are largely relayed by the sensory cranial nerves to the principal nucleus of the trigeminal nerve, which projects to the ventral posteromedial nucleus, then to higher order brain regions. Vibration detected by cutaneous mechanoreceptors along the body is mostly sent to the dorsal column of the spinal cord to synapse on the dorsal column nuclei, relayed to the ventral posterolateral nucleus in the thalamus and sent to multiple targets in higher brain regions. Instead of an exhaustive literature review on how touch is processed in vertebrates, this section will focus on potential pathways for vibration detection in lepidosaurs.

The principal trigeminal nucleus and the descending trigeminal nucleus receive sensory input from the trigeminal nerve of lizards, and project to the ventral thalamus, similar to other vertebrates (ten Donkelaar et al., 1987). The principal trigeminal nucleus and the descending trigeminal nuclei in snakes are also referred to as the common sensory trigeminal nuclei, to differentiate from the infrared-processing lateral descending nucleus, but otherwise follow the lizard pattern (Molenaar & Fizaan-Oostveen, 1980). Snakes have mechanoreceptors in their cephalic region termed sensilla or corpuscles that project beyond the surface of the scales (e.g. Crowe-Riddell et al., 2019; Jackson, 1977). The ophidian sensilla bears a superficial resemblance to the better-known crocodilian integumentary scale organs

(Jackson, 1977; Jackson & Sharawy, 1980; Leitch & Catania, 2012). Recent work has shown that the crocodilian integumentary scale organ is a true multi-modal receptor (Di-Poï & Milinkovitch, 2013); the same resolution has yet to be applied to the ophidian scale sensillae. The scale sensillae of several groups of aquatic/marine snakes have been proposed to respond to aquatic vibrations (Povel & Van Der Kooij, 1997; Westhoff et al., 2005) though this functional role has not been well established. Cephalic corpuscles of terrestrial forms has been shown to respond to 256 Hz vibrational stimuli, however details of their tuning are unclear (Jackson & Doetsch, 1977b, 1977a). Additionally, tentacled snakes (*Erpeton tentaculatus*) have a distinctive and unique pair of facial appendages that protrude from the rostral margins of the head. While lacking scale sensilla, the appendages are innervated by the trigeminal nerve. They are used to detect disturbances in the water and are sensitive to 50-150 Hz vibrations (Catania et al., 2010).

Body mechanoreceptors may also mediate vibration detection. In lizards, primary afferent fibers of the dorsal root ascend via the dorsal column to reach the dorsal column nuclei, which in turn project via the medial lemniscus to mainly the ventrolateral region of the thalamus, not dissimilar to mammals (ten Donkelaar et al., 1987). The dorsal column system in snakes is reduced (Ebbesson, 1969).

A particularly noteworthy set of studies concerns the issue of “somatic hearing” involving potentially similar or parallel pathways in the torus semicircularis. When Hartline & Campbell (1969) recorded in the torus semicircularis of the snake midbrain, they found that different snake species were all sensitive to sound or vibration delivered to areas along the body, extending beyond midsection, indicating

a mechanism by which snakes could detect peripheral vibrations. This led them to probe the neural basis for snake vibration detection. Hartline & Campbell (1969) transected the spinal cord of *Pituophis* and obtained midbrain responses when sounds of 140 Hz at 80 dB SPL were delivered. These midbrain responses were abolished after bilateral destruction of the inner ear, even when stimuli increased to 100 dB SPL. In another species, *Crotalus viridis*, severing of the spinal cord also did not eliminate responses to mid-body sound and vibration stimulation. The authors concluded that the spinal cord does not contribute to sound or vibration detection in snakes. However, their experiments only disproved the necessity of the spinal cord for midbrain responses. Without eliminating contributions of the inner ear and recording with only the spinal cord intact, the sufficiency of spinal cord input cannot be ruled out.

Perhaps the authors realized the fallacy in their conclusion. Hartline (1971b) explored the responses of the torus to sound and vibration by addressing the inner ear and the spinal cord separately. Contrary to the previous report (Hartline & Campbell, 1969), he found that the spinal cord, or somatosensation, could mediate responses in the auditory midbrain. The response thresholds to sound pressure and substrate vibration after surgical isolation of the somatic pathways were generally greater than the response thresholds following surgical isolation of the inner ear pathways. For the somatic system, the shape of the response threshold curve was flatter without a distinct best frequency. Stimuli delivered directly to the head or stapes failed to elicit a response, suggesting that the somatosensory responses in the midbrain are imparted by input to the spinal cord, instead of cranial nerves. The topography of sites from

which a somatic response could be recorded also differed from that of the auditory system. In a follow-up paper, Hartline (1971a) further demonstrated that the latency of the somatic system was consistently (as many as 10ms) lower than that of the auditory system. Mechanoreceptors such as cutaneous vibration receptors found in Proske (1969b) may serve as peripheral detectors, however systematic differences of intensity-latency curves at different frequencies indicate a multi-receptor contribution (Hartline, 1971a). Hartline (1971b) noted that recordings sites yielding auditory responses were restricted to the caudomedial region of the midbrain, while somatic response sites occupied a larger area in the horizontal plane. Somatic areas were also more superficial than auditory areas.

The cytoarchitecture of the snake torus semicircularis differs considerably from that of its lizard relatives (Molenaar, 1976; Senn, 1969, 1979; Senn & Northcutt, 1973). In other reptiles, including most lizards, the central nucleus of the torus semicircularis forms the main mass of the caudal torus semicircularis. In contrast, the caudal part of the snake torus semicircularis occupied by the “paratorus”, and the central nucleus of the torus semicircularis forms only a thin layer ventral to the “paratorus”. In more rostral sections of the midbrain, the “paratorus” merges with the nucleus lateralis profundus mesencephali, lateral to the periventricular central nucleus. Considering the disproportional sizes of the “paratorus” and the central nucleus, and their respective locations in the midbrain, the somatosensory responses, and possibly the auditory responses, recorded by in Hartline (1971b, 1971a) likely was recorded from the “paratorus”.

While the terminology “paratorus” has been only used by Senn and colleagues to discuss the midbrain in snakes (Senn, 1969), and later in some burrowing lizards in the context of snake evolution (Senn & Northcutt, 1973), an analogous midbrain structure that receives input from spinal pathways has been described in other vertebrates. For example, in lizards, there is a superficial nucleus or intercollicular nucleus external to the central nucleus of the torus semicircularis (Browner & Robinson, 1977; Foster & Hall, 1978; Kennedy & Browner, 1981), that receives ascending somatosensory input from the spinal cord (Foster & Hall, 1978). Recordings from turtles (Khachunts, 1982) also report a relatively superficial region of the torus (termed intercollicular nucleus) that responded primarily to somatosensory stimuli and was layered over a central region that primarily responds to auditory stimuli. A midbrain somatosensory area with similar relative locations has also been identified in crocodilians (Pritz & Stritzel, 1989) and birds (Wild, 1995). In cats (Aitkin et al. 1978; Aitkin et al. 1981), both electrical stimulation to the dorsal column and acoustic stimuli to the ear were presented while recording from the external nucleus of the inferior colliculus (ICX). Considering similarities in response properties and neuroanatomical evidence, it is possible that similar structures to the snake paratorus are found in other vertebrate species.

Many questions remain regarding the snake paratorus. In some species, the midbrain somatosensory area also receives input from auditory nuclei (Aitkin et al., 1978, 1981; Belekova et al., 1985; Wild, 1995), whereas in others input from the auditory hindbrain is absent (Foster & Hall, 1978; Pritz & Stritzel, 1989; Yamamoto et al., 2010). In Hartline (1971a, 1971b), it is unclear whether the auditory responses

were recorded in the paratorus or the central nucleus. Additionally, the relative size paratorus in snakes is considerably larger than the somatosensory midbrain area in other vertebrate species, and its functional significance is unknown. Moreover, the possible connections of the snake paratorus have only been speculated based on observation in Nissl material (Senn, 1969).

## Chapter 2: Central projections of auditory nerve fibers in the western ratsnake (*Pantherophis obsoletus*)

This chapter is comprised of previously published work: Han, D., & Carr, C. E. (2023). Central projections of auditory nerve fibers in the western rat snake (*Pantherophis obsoletus*). *Journal of Comparative Neurology*, 531(12), 1261-1273.

### 1. Introduction

With over 3800 species in the suborder Serpentes, snakes are a diverse group of reptiles that have invaded ecological niches ranging from the oceans to tropical canopies (Uetz et al., 2021). Little is known about their auditory systems, except that they lack tympana and are instead thought to detect vibration through the lower jaw (Wever, 1978), which articulates with the quadrate coupled to the inner ear via the stapes (Figure 2.1a). How snakes process this vibrational information is poorly understood, motivating our study of the auditory nerve projections to brain.

Among sauropsids, auditory brainstem circuitry has been most extensively studied in birds, and their organization is thought to be representative of all groups in this clade (Carr et al., 2016; Carr & Konishi, 1990; Takahashi & Konishi, 1988b, 1988a; Walton et al., 2017). The auditory branch of the VIIIth nerve terminates in two nuclei, a more caudal nucleus magnocellularis (NM), and a rostral nucleus angularis (NA). NM and NA serve as the first-order cochlear nuclei for ascending binaural and monaural pathways to the inferior colliculus (Ashida & Carr, 2011; Grothe et al., 2004).

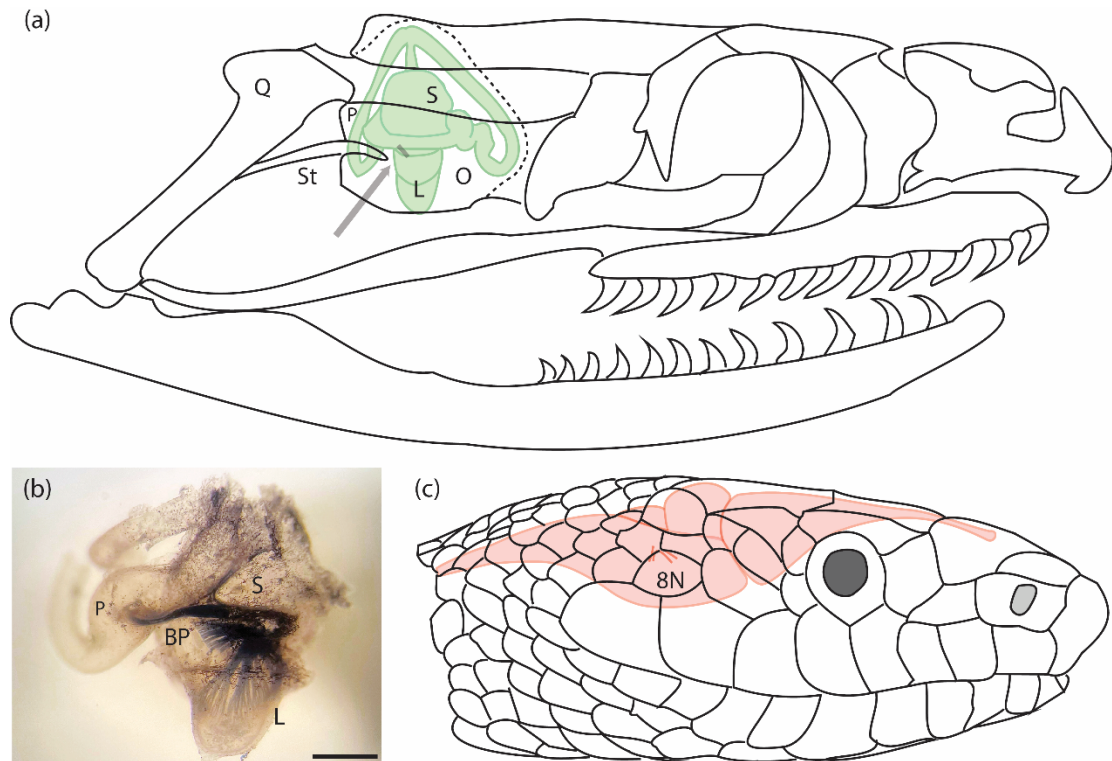


**Figure 2.1 Overview of auditory structures of the western ratsnake**

(a) Schematic line drawing of the skull of the western ratsnake showing the relative position of the inner ear (green). The rostral border of the otic capsule is marked by the dashed line. Grey arrow points to the basilar papilla located close to the medial wall of the membranous labyrinth. L, lagena; O, otic capsule; P, posterior semicircular canal; Q, quadrate; S, sacculus; St, stapes. Line drawing of the ratsnake skull adapted from Auen & Langebartel (1977).

(b) Medial view of the posterior branch of the VIIIth nerve innervating the basilar papilla (BP), lagena macula (L) and posterior crista (P). The nervous tissue was stained with Sudan black B. The saccular macula (S) is on the opposite side (lateral) and occluded from view. Scale bar = 1 mm.

(c) Schematic line drawing of the head of the western ratsnake and the relative position of the brain (pink). 8N, cranial nerve VIII.

Prior studies of the snake auditory system have shown that the auditory portion of the VIIIth nerve targets dorsal nuclei in the rostral medulla, although interpretation and description of its first order projections vary. Holmes (1902) described a single “small and ill-defined” nucleus dorsalis. Weston (1936) described a single cochlear nucleus homologous to NA, while ten Donkelaar & Nieuwenhuys (1979) found only a “dorsal magnocellular nucleus”. Schwab (1979) confirmed a single cochlear nucleus but noted that in *Cylindrophis* it could be divided into anterior and posterior subnuclei. Molenaar (1976), while examining *Python reticulatus*, identified a small rostral NA in addition to a large caudal NM. Miller (1980) also differentiated NM and NA, and further separated NM into nucleus magnocellular medialis and nucleus magnocellularis lateralis to align with the nuclear subdivisions of the caiman (Leake, 1974) but noted that in some species NA was not “sharply demarcated” and the two magnocellular nuclei may be merged. These results were agreed upon by DeFina (1981) and Defina & Kennedy (1983).

There are several potential explanations for the inconsistencies in the description of snake cochlear nuclei. First, most literature recognized snake cochlear nuclei based on histological boundaries, which may be confounded when nuclei lack differentiation, a phenomenon sometimes observed in the octavolateralis system (McCormick, 1999). Second, snakes in general have small basilar papillae (Miller, 1968, 1978; Wever, 1978). *In vivo* recordings show auditory sensitivities below 1000 Hz, which is a restricted low-frequency bandwidth compared with most other sauropsids (Christensen et al., 2012; Hartline, 1971b; Wever, 1978), suggesting that

snake cochlear nuclei may be reduced and potentially difficult to find. Third, snakes lack a tympanum and are therefore most sensitive to substrate vibration (Hartline, 1971b; Hartline & Campbell, 1969; Wever, 1978). They detect high-amplitude sound pressure via sound-induced head vibrations (Christensen et al., 2012).

In this study, we used tract tracing, immunohistochemistry and Nissl staining to investigate the projections of the cochlear branch of the VIIIth nerve in the western ratsnake (*Pantherophis obsoletus*). We hypothesized that there are dedicated auditory nuclei in the brainstem similar to that seen in other sauropsids, and that inconsistencies in the literature might reflect species-specific variation. The western ratsnake was selected due to its wide availability, indications of distinct cochlear nuclei (DeFina, 1981; Defina & Kennedy, 1983), moderate hearing abilities (Wever, 1978), and well-described inner ear (Miller & Beck, 1990). In addition, Wever's (1978) methods of using cochlear microphonics to assess hearing thresholds have received criticism (Manley, 2017), so we re-evaluated auditory sensitivity of the western ratsnake using vibration evoked potential recordings. We found the western ratsnake was most sensitive to low frequencies, similar to other snakes. Further, we showed that the auditory nerve of the western ratsnake projects to two first-order cochlear nuclei, a small caudomedial NM and a larger, distinct rostralateral NA.

## 2. Material and Methods

A total of 37 western ratsnakes (*Pantherophis obsoletus*, snout-vent length 20-140 cm) were obtained commercially for this study. Snakes were kept in a 12:12-hour light-dark cycle with 21-27 °C temperature range and offered mice and water ad

libitum. Anesthesia and animal handling protocols were approved by the University of Maryland College Park Animal Care and Use Committee.

## 2.1 Vibration evoked potential recordings

Twelve snakes were used for vibration evoked potential recordings. Snakes were anesthetized with isoflurane (5%) until fully anesthetized (determined by loss of muscle tone, lack of righting reflex, and lack of reflex to tail pinch), and plane of anesthesia was maintained with 2% isoflurane for the duration of the experiment. Experiments were performed in an anechoic chamber (21°C). We used QuickABR, a custom software developed by Christian Brandt (University of Southern Denmark, Odense, Denmark), to generate stimuli and record evoked potentials (Brandt et al., 2007). Vibrational stimuli were produced with a mini-shaker (Type 4810; Brüel & Kjær, Nærum, Denmark) connected to a custom-made platform and placed under the snake's head (Figure 2.2a; Christensen et al., 2012). The mini-shaker was calibrated using a piezoelectric accelerometer (Type 4381; Brüel & Kjær, Nærum, Denmark) connected to a conditioning amplifier (Type 1704; Brüel & Kjær, Nærum, Denmark). The accelerometer itself was calibrated using a calibration exciter (Type 4294; Brüel & Kjær, Nærum, Denmark) at  $10 \text{ ms}^{-2}$ , 159.15 Hz.

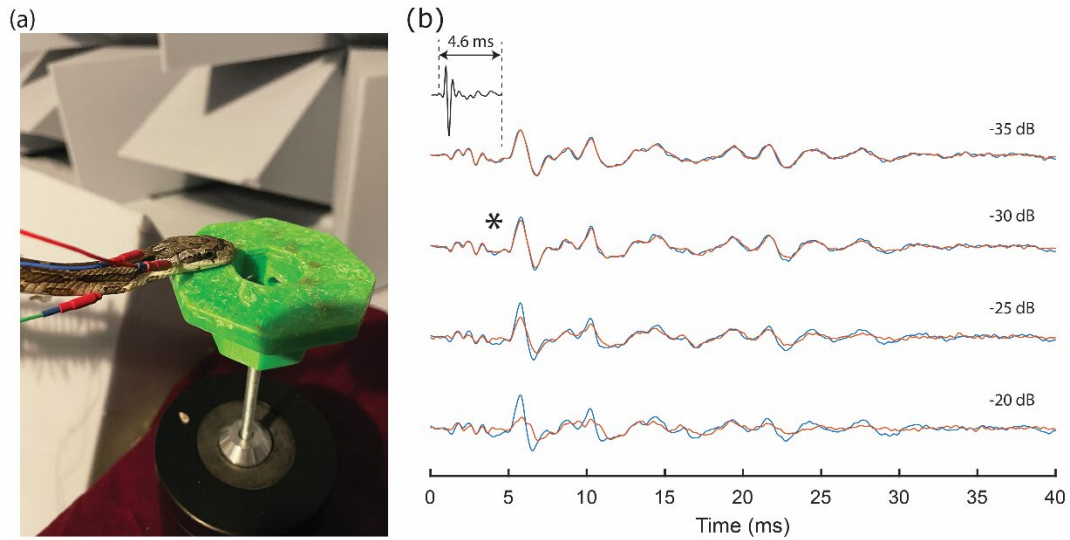


**Figure 2.2 Vibration evoked potential recordings**

(a) Recording setup for measuring evoked potentials. The snake's head was placed on the green platform connected to a mini shaker. Two recording electrodes were inserted on the snake's head, one dorsal to the brainstem (blue) and one dorsal to the VIIIth nerve (red). The reference electrode (green) was inserted into the neck of the snake.

(b) Vibration evoked potential waveforms in response to click stimulations in the presence of a 100 Hz masker. Responses to masked (orange) and unmasked (blue) clicks were overlaid, and threshold was determined visually as the lowest level where a clear discriminable difference could be seen from the two waveforms (indicated by the asterisk). Click stimulus is shown above evoked potential waveforms in black.

Vibrational stimuli and experimental procedure for measuring vibration evoked potential recordings followed that of Capshaw et al. (2020). In brief, we compared evoked potentials in response to a broadband click with or without a masking tone, and detection thresholds were determined visually as the lowest level where the tone had a masking effect (Figure 2.2b). Compared to using tone bursts, this method avoids the “frequency splatter” of short low-frequency stimuli (Brandt et al., 2018). The click stimulus used consisted of one half-cycle of a 2000 Hz signal at an amplitude that elicited 90% of the maximal neural response, and pure tone maskers ranged from 20-1000 Hz. Three electrodes were inserted subcutaneously, including two measuring electrodes, one dorsal to the brainstem and one dorsal to the VIIIth cranial nerve, and one reference electrode placed into the neck of the snake. Evoked potentials were amplified with a RA4PA Medusa pre-amplifier (Tucker Davis Technologies, Alachua, FL, USA) and recorded by an RM2 digital signal processor (Tucker Davis Technologies, Alachua, FL, USA) with a sampling rate of 24.4 kHz. Evoked potentials were averaged over 800 repetitions, and every second stimulus was phase inverted. Vibration thresholds were plotted against frequency in MATLAB v2019b (Mathworks, Natick, MA, USA).

## 2.2 Tract tracing

A total of 19 snakes were used for tract tracing. Following the anesthesia described above, the heart was exposed by ventrally opening the body cavity, and the snakes were perfused transcardially with oxygenated ice-cold Ringer’s solution (96.5mM NaCl, 31.5mM NaHCO<sub>3</sub>, 4mM CaCl<sub>2</sub>, 2.6mM KCl, 2mM MgCl<sub>2</sub>, and

20mM D-glucose, from Kohl et al. (2014)). After decapitation and removal of the lower jaw, the dorsal skull was placed in a Sylgard-lined dish containing ice cold Ringer's solution.

To locate the auditory endorgan, the basilar papilla, and its associated nerve bundle in the inner ear labyrinth, we first stained the inner ear with Sudan black B, modified from procedures of Rasmussen (1961). Briefly, the whole head was immersion-fixed in 4% paraformaldehyde in 0.01M phosphate-buffered saline (PBS) overnight. The membranous labyrinth of the inner ear was carefully dissected out and washed in distilled water to remove excess fixative. The tissue was rinsed in 30%, 50% and 70% ethanol for 5 minutes each, then transferred to a saturated alcoholic solution of Sudan Black B. After staining for 10 minutes, the tissue was transferred to 70% ethanol for differentiation under the dissection scope, then washed in 50% ethanol and distilled water. Finally, the tissue was transferred to 90% glycerol for clearing and storage.

To label afferents from the basilar papilla, the otic capsule was opened dorsally to expose the membranous labyrinth. After removal of otoconia in the saccule, the saccular macula was moved aside, and the cochlear duct opened with fine forceps to reveal the small bundle of nerves supplying the basilar papilla. Excess fluids were removed, and biotinylated dextran amine (BDA; Dextran, Biotin, 3000MW, Lysine Fixable, Invitrogen, Carlsbad, CA) crystals were applied to the papillar nerve bundle with a tungsten electrode. The isolated head was placed in a humid chamber in an 8-10°C refrigerator to allow tracer uptake for 15 minutes, and then rinsed multiple times with Ringer's solution to remove excess tracer.

After injection of tracer into the basilar papilla, the isolated head was immersed in oxygenated Ringer's solution in an 8-10°C refrigerator for 24- 48 h to allow for tracer transport, with oxygenated Ringer's solution changed every 12-24 h. The brain was then immersion-fixed in 4% paraformaldehyde in 0.01M PBS for 24 h, and subsequently cryoprotected in 30% sucrose in 0.01M PBS for 24 h. Tissue was cut on a freezing microtome in 50 µm coronal sections, rinsed in 0.01M PBS, and then incubated in avidin biotin complex (ABC; Vector Laboratories, Burlingame, CA), in 0.2% Triton X in 0.01M PBS solution for 12 h at room temperature. Afterwards, sections were reacted using Vector SG/Peroxidase Substrate Kit (Vector Laboratories, Burlingame, CA) for 5-10 min, rinsed with buffer, and then mounted on gelatin-subbed slides. After air-drying overnight, slides were counterstained with Neutral Red, dehydrated and cover slipped. In specimens used for basilar papillae label, the membranous labyrinths were also dissected free, reacted and mounted to check for possible contamination of tracer in other endorgans.

### 2.3 Immunohistochemistry

A total of three snakes were used for immunohistochemistry studies. After fixing and cryoprotecting the brain as described above, the brains were sectioned on a freezing microtome at 30 µm coronal sections. Free-floating sections were incubated in a blocking solution of 4% normal goat serum in 0.01M PBS with 0.4% Triton-X (TX) for 20 minutes. Sections were subsequently incubated in antisera against 1) calbindin (CB; Swant, Belinzona, Switzerland) and 2) parvalbumin (PV; Sigma-Aldrich, St. Louis, MO, USA), both diluted at 1:1000 in 0.01M PBS and 0.02% TX,

for 18 hrs at 4 °C (Yan et al. 2010). After washing, sections were incubated in biotinylated goat anti-rabbit secondary antisera, diluted 1:1000 in 0.01MPBS and 0.02% Triton-X, for 1 hour. Following wash, sections were then incubated in ABC and reacted with the SG/peroxidase kit and mounted on gelatin subbed slides to air-dry overnight. Slides were then counterstained with Neutral Red, dehydrated and coverslipped. In a previous study using the same antibodies, western blots were used to examine antibody specificity in the related tokay gecko (Yan et al, 2010). In the gecko, blots with CB and PV showed a major band at an estimated molecular weight of 28 kD and 15 kD, respectively, similar to the measurement in other species (CB: Ellis et al., 1991; PV: Celio, 1990; Lohmann and Friauf, 1996).

To optimize staining patterns, we incubated cerebellar tissue in the presence or absence of primary antibodies. Purkinje cells were immunoreactive to CB and PV primary antibodies, and were not stained in their absence (Basianelli, 2003). Serial dilutions of primary antibodies from 1:500 to 1:5000 were used to optimize the working concentration of each antibody. We measured the optical density of the labelled neurons in relation to antibody dilution, and selected an antibody concentration of 1:1000 because it was in the linear stage of the optical density vs concentration plot.

## 2.4 Histology

Two snakes were processed for Nissl staining. After fixation, cryoprotection and sectioning of the brain as described above, sections were mounted on gelatin-subbed slides and air-dried overnight. Slides were then stained with cresyl violet,

dehydrated and coverslipped. A third snake was used for plastic embedding in order to visualize myelinated auditory nerve fibers. After anesthesia, this snake was perfused transcardially with 2% electron microscope (EM) grade paraformaldehyde and 2% EM grade glutaraldehyde in 0.13M phosphate buffer, pH 7.4. Following 24 h post-fix of the snake's head, the brain was removed and sectioned with a vibratome at 100  $\mu$ m. The basilar papilla and its connecting nerve were also carefully removed. The tissue was fixed in 1% osmium tetroxide, washed with 0.05M maleate buffer, pH 5.2, dehydrated and embedded in Epon araldite 502 (Electron Microscopy Sciences, Hatfield, PA, USA). The papillar nerve containing preganglionic fibers was sectioned orthogonal to the nerve root at 1  $\mu$ m thickness, and the brain and postganglionic fibers were sectioned in the transverse plane of the brain, oblique to the long axis of the axons. Sections were mounted on glass slides and stained with toluidine blue.

## 2.4 Anatomical analysis

Brainstem auditory nuclei were identified based on physical location and projection pattern. Labelled neurons, terminals and axons were visualized, photographed, and traced with Neurolucida (MBF Bioscience, Williston, VT) in conjunction with a light microscope (Olympus BX60). Nuclear volume and morphometrics of neurons and terminals were quantified with Neurolucida Explorer (MBF Bioscience, Williston, VT). From BDA labelled sections, random non-overlapping locations were selected within the borders of the cochlear nuclei and the area of every terminal in each region of interest was measured at 1000x.

The number of neurons was counted by first drawing a contour around the cochlear nucleus, and then placing a marker on every neuron with a clear nucleolus within the nuclear boundaries, thereby counting every neuron. We counted the total population of neurons instead of obtaining an estimate using stereological protocols because the volume of NM was small. To evaluate the feasibility of using a stereological estimate, we first calculated the average sampling fraction (asf) using the equation  $asf = \text{area of counting frame} / \text{area of sampling grid}$ . In a given section, the total area of NM often fits within the size of the counting frame, and the area of the counting frame and the area of the sampling grid are similar in size. Hence, in the case of counting NM neurons, asf is approximately 1, requiring sampling of 100% of each section. In other words, the total population of NM neurons must be counted instead of estimated from stereology. To ensure the same methods were used for counting NM and NA, we decided to count, instead of estimate, the total population of NA neurons. We were aware that errors arising from split nucleoli might cause overestimation and should be corrected by an equation from Abercrombie (1946):  $N_{real} = N_{counted} \times T/(T+h)$ , where T is the thickness of the section, and h is the thickness of the nucleoli. However, since the section thickness was 50  $\mu\text{m}$ , which greatly exceeds the typical maximum dimension of nucleoli,  $T/(T+h)$  approaches 1.

Morphometrics of neurons were measured at the plane where a clear nucleolus could be seen. The form factor of neurons was defined as the area of the neuron profile divided by the area of a circle having the same perimeter, calculated as  $(4\pi \times \text{area} / \text{perimeter}^2)$ . As the contour of the neuron approaches a perfect circle, the value approaches 1. The total number of auditory nerve fibers was counted by placing

a marker on every axon in a single cross-section of the preganglionic papillar nerve. The mean diameter of preganglionic fibers was quantified by measuring cross-sections of every axon in MyelTracer (T. Kaiser et al., 2021) as equivalent circle diameter (ECD), calculated from  $2\sqrt{(\text{area}/\pi)}$ . While the plane of section was orthogonal to the nerve root, note that use of ECD may overestimate the axon diameter if the plane of section deviates from a perfect cross section. Postganglionic fiber diameters were obtained from transverse sections that contained obliquely cut fibers, and in these cases the largest diameter of the short axis was measured in ImageJ (NIH). Measurements of the post-ganglionic axon diameter were obtained from all visible axons in a single 1  $\mu\text{m}$  semi-thin transverse brain section. The g-ratio was calculated as the ratio between the inner axon diameter and the outer axon diameter that included myelin.

### 3. Result

#### 3.1 Vibrogram

The vibration thresholds of western ratsnakes followed a U-shaped curve ( $n = 5$  for 20 Hz and 35 Hz stimuli,  $n = 12$  for all other frequencies tested; Figure 2.3), with best frequencies between 50 Hz ( $-29.6 \pm 2.4$  dB re.  $\text{ms}^{-2}$ ) and 100 Hz ( $-29.2 \pm 3.2$  dB re.  $\text{ms}^{-2}$ ). The latency of the evoked potential response from the click stimulus onset was  $4.6 \pm 0.8$  ms at  $21^\circ\text{C}$  ( $n = 12$ , Figure 2.2b). Vibration sensitivity was similar for 20 Hz and 35 Hz, increased at 50 Hz and 100 Hz, and declined rapidly at frequencies above 100 Hz. All thresholds measured were at least 20 dB above the total octave noise.

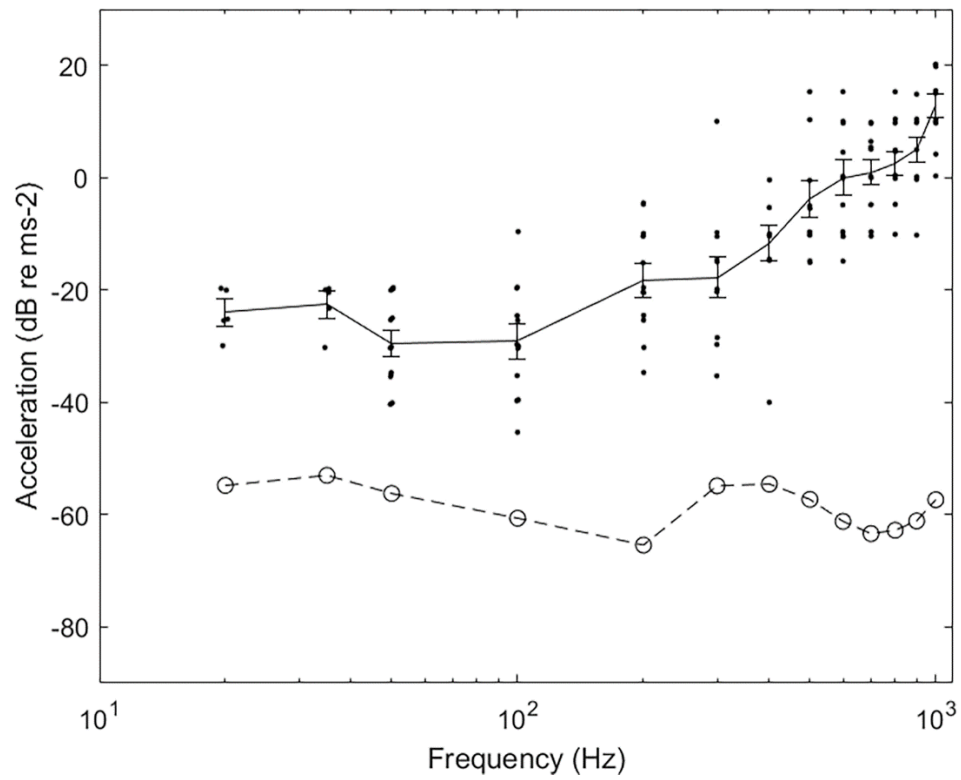


**Figure 2.3 Vibrogram of the western ratsnake**

Detection thresholds are plotted as mean  $\pm$  SEM. Individual thresholds ( $\bullet$ ); noise floor ( $\circ$ ). For 20Hz and 35 Hz, N=5. For all other frequencies tested, N=12.

### 3.2 Auditory nerve

The VIIIth nerve is divided into an anterior branch that receives input from the anterior and lateral semicircular canals and the saccule and utricle, and a posterior branch that receives input from the posterior semicircular canal, saccule, basilar papilla, and lagena (see Figure 2.1b, 2.1c; Baird, 1970; Wever, 1978). The basilar papilla is recognized as an auditory endorgan in land vertebrates (Fritzsche et al., 2013; Walton et al., 2017). BDA crystals placed in the small nerve roots supplying the basilar papilla labelled cell bodies in the dorsal portion of the posterior ganglia. In the brain, the labeled axons in the posterior nerve root bifurcated to form a lateral ascending branch projecting to NA and a more medial descending branch projecting to NM, with NA extending more rostrally and NM extending more caudally (Figure 2.5a, 2.5b). Cochlear nerve fibers projecting to NM mainly travelled ventral to NA. Fibers from the basilar papilla did not project to other hindbrain targets.

We counted 838 fibers from the cross-section of the auditory nerve (Figure 2.4a). Table 2.1 summarizes the measurements of axon diameters leaving the basilar papilla (Figure 2.4a; preganglionic), leaving the cochlear ganglia before bifurcation (Figure 2.4b; postganglionic, pre-bifurcation) and after bifurcation to innervate the NA (Figure 2.4c) and NM (Figure 2.4d), as well as their respective g-ratios. The sites of bifurcation were not visible in the semi-thin sections. We also could not accurately identify the boundaries of NM. Thus, identification of the post-bifurcation fibers was an approximation based on their location in the transverse brain sections. Note that post-ganglionic fibers were measured from transverse sections of the brain and typically have more variable diameters.

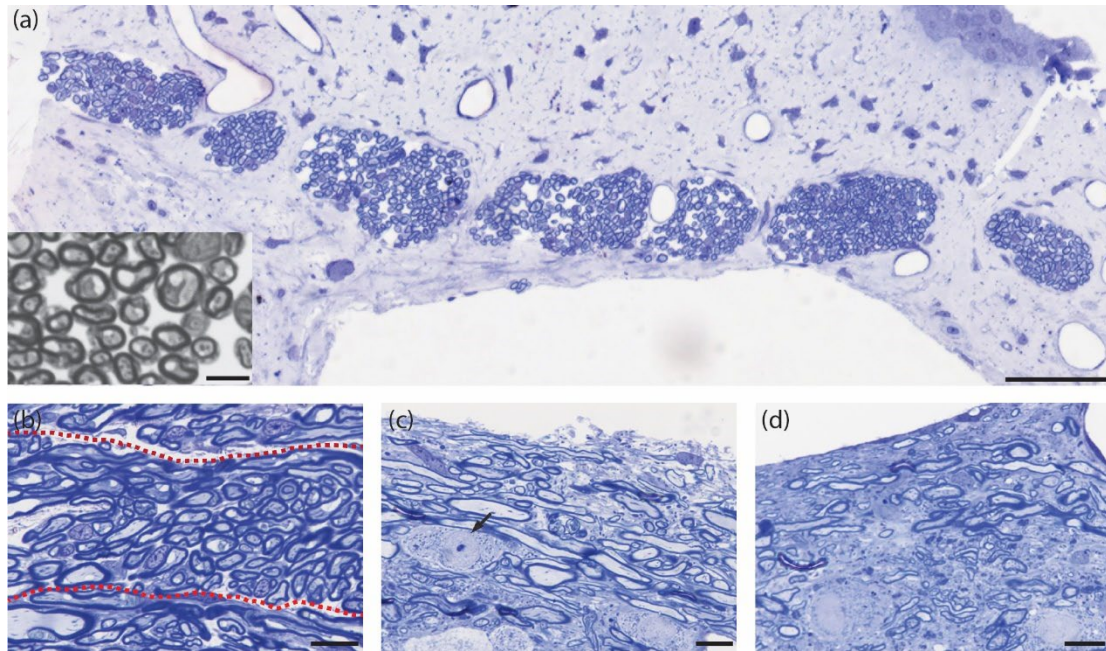


**Figure 2.4 Auditory nerve axons**

- (a) Low magnification cross-sections of the pre-ganglionic papillar nerve. For all photomicrographs, the left side shows medial, right side lateral, top dorsal, and bottom ventral orientation. Scale bar = 50  $\mu\text{m}$ . Inset grey-scale higher magnification view shows that most fibers are myelinated. Scale bar = 5  $\mu\text{m}$ .
- (b) Transverse brain sections showing the post-ganglionic papillar nerve in the dorsal part of the posterior branch of the VIIIth nerve (area in dashed lines). Scale bar = 10  $\mu\text{m}$ .
- (c) Transverse brain sections showing the papillar nerve collaterals in NA. Arrow indicates an NA neuron. Scale bar = 10  $\mu\text{m}$ .
- (d) Transverse brain sections showing the possible papillar nerve collaterals around NM. Scale bar = 10  $\mu\text{m}$ .

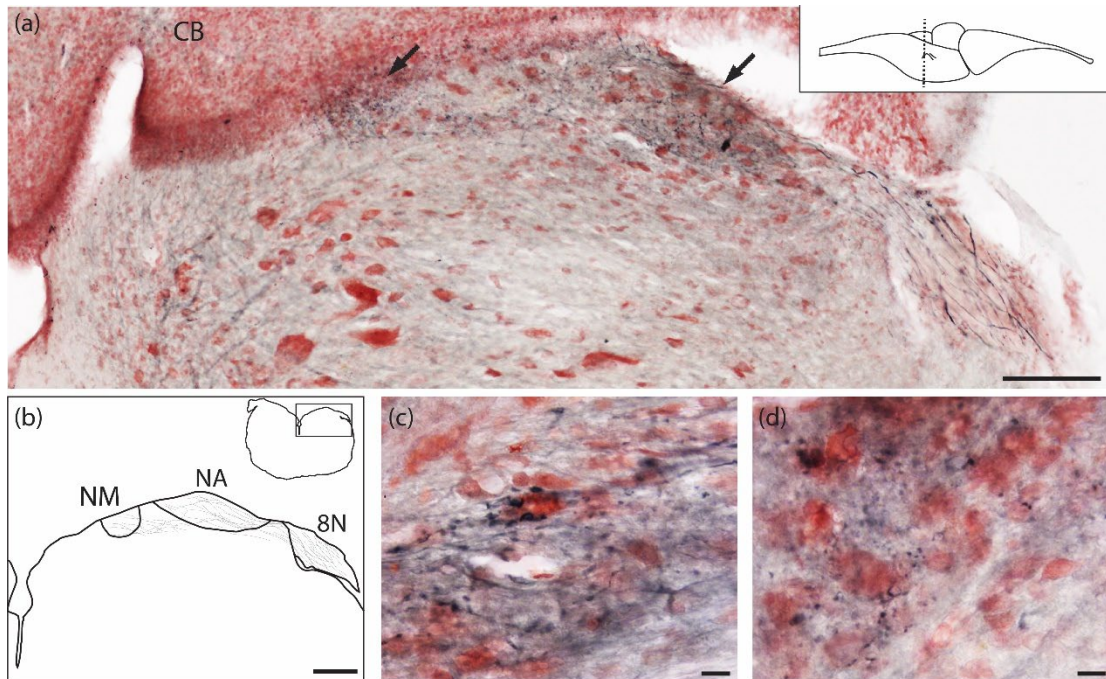


**Figure 2.5 Projections of the papillar branch of the VIIIth nerve**

(a) Brainstem projections of the auditory branch of the VIIIth nerve. Arrows denoting two terminal fields in NM (left) and NA (right). A small portion of the dorsal brainstem overlaps the cerebellum (CB). Inset: Lateral view showing plane of section. Scale bar = 100  $\mu$ m.

(b) Schematic line drawing of cochlear nuclei in based on projections in (a). Axons were labelled in grey to show their projection to the cochlear nuclei. 8N, cranial nerve VIII. Scale bar = 100  $\mu$ m.

(c) Terminal field in NA. Scale bar = 10  $\mu$ m.

(d) Terminal field in NM. Compared to the terminal field in NA, most of the bouton terminals were small in NM. Scale bar = 10  $\mu$ m.

| Location                                 | Number of fibers | Plane of section relative to long axis | Measurement type           | Mean diameter (excluding myelin) $\pm$ S.D. | Mean diameter (including myelin) $\pm$ S.D. | Mean g-ratio $\pm$ S.D. |
|------------------------------------------|------------------|----------------------------------------|----------------------------|---------------------------------------------|---------------------------------------------|-------------------------|
| Pre-ganglionic                           | 791              | Perpendicular                          | Equivalent circle diameter | $2.2 \pm 0.6$                               | $3.3 \pm 0.7$                               | $0.66 \pm 0.07$         |
| Post-ganglionic, pre-bifurcation         | 150              | Oblique                                | Minimal diameter           | $1.9 \pm 0.8$                               | $3.0 \pm 0.9$                               | $0.63 \pm 0.09$         |
| Post-ganglionic, post-bifurcation, to NA | 120              | Oblique                                | Minimal diameter           | $1.4 \pm 0.8$                               | $2.0 \pm 0.8$                               | $0.67 \pm 0.11$         |
| Post-ganglionic, pre-bifurcation, to NM  | 150              | Oblique                                | Minimal diameter           | $1.0 \pm 0.5$                               | $1.5 \pm 0.6$                               | $0.68 \pm 0.09$         |

**Table 2.1 Diameter of axons from the basilar papilla**

All measurements of fiber diameters (in  $\mu\text{m} \pm \text{S.D.}$ ) obtained from semi-thin sections viewed with a light microscope at 1000x. Preganglionic fiber diameters were quantified from the cross-section of the auditory nerve, while all other measurements were sampled from transverse sections of the brain. Due to the difficulty of discerning cochlear nuclei in the semi-thin sections, identification of the fibers was based on their approximate location.

After labeling of the basilar papilla with BDA, bouton terminals were found to be distributed throughout NA and NM. Most bouton terminals were located in the neuropil, with a smaller number of large perisomatic bouton terminals. The mean area of bouton terminals in NA was  $2.3 \pm 1.3 \mu\text{m}^2$  ( $n = 234$ , from 3 snakes) and the mean area of bouton terminals in NM was  $1.4 \pm 1.0 \mu\text{m}^2$  ( $n = 287$ , from 3 snakes). No large lobulated terminals (endbulbs) were observed in the western ratsnake NA or NM.

### 3.3 Nucleus angularis

NA formed a distinguishable eminence on the dorsal surface of the medulla adjacent to the VIIIth nerve root (Nissl, Figure 2.6a). Caudally NA extended as a thin layer that impinged upon the ventrally located nucleus vestibularis ovalis (VeO) and expanded rostrally to end near the cerebello-medullary junction. NA axons, terminals and neurons were strongly PV-positive, and the PV-positive labeled area overlapped with label from papillar nerve projections (Figure 2.6b). In cresyl violet stained sections, NA contained large round cells (Figure 2.6c), small round cells (Figure 2.6d), and fusiform cells (Figure 2.6e). Measurements of cell morphometrics are compiled in Table 2.2. The cell areas of the large round cell type in NA had a large standard deviation, suggesting that this category may be heterogeneous. We did not quantify the respective percentages of each neuron type, but observed that round cells were relatively abundant, whereas fusiform cells were relatively scarce. We counted  $580.3 \pm 54.3$  neurons in one side of NA (average from 3 animals).

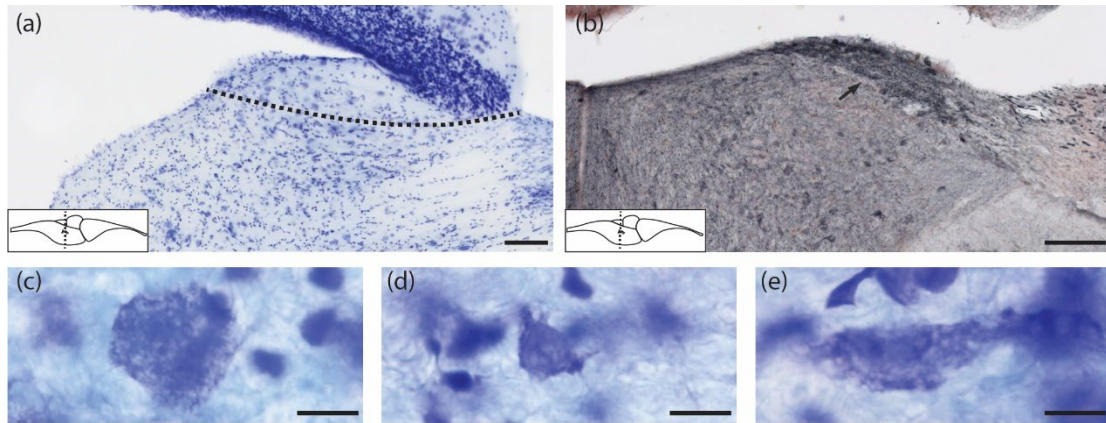


**Figure 2.6 Nucleus angularis in the western ratsnake**

- (a) Location and boundaries (dashed line) of NA in the acoustic tubercle, stained with cresyl violet. Inset: Lateral view showing plane of section. Scale bar = 100  $\mu\text{m}$ .
- (b) PV-ir NA neurons and neuropil (arrow), counterstained with neutral red. Scale bar = 100  $\mu\text{m}$ .
- (c)-(e). High magnification images of Nissl-stained large round (c), small round (d) and fusiform (e) cells in NA. Scale bar = 10  $\mu\text{m}$ .

| Cell type                      | Sample size | Mean area $\pm$ S.D. | Form factor $\pm$ S.D. |
|--------------------------------|-------------|----------------------|------------------------|
| <b>Nucleus angularis</b>       |             |                      |                        |
| Large round                    | 32          | 142.1 $\pm$ 71.2     | 0.82 $\pm$ 0.07        |
| Small round                    | 30          | 39.8 $\pm$ 10.6      | 0.85 $\pm$ 0.04        |
| Fusiform                       | 9           | 79.1 $\pm$ 34.7      | 0.52 $\pm$ 0.06        |
| <b>Nucleus magnocellularis</b> |             |                      |                        |
| Round                          | 51          | 64.7 $\pm$ 22.4      | 0.81 $\pm$ 0.07        |
| Fusiform                       | 15          | 76.4 $\pm$ 26.3      | 0.54 $\pm$ 0.08        |

**Table 2.2 Morphometrics of different neuron types**

Measurements of NA neurons obtained from Nissl-stained sections from a single animal. Measurements of NM neurons obtained from CB-ir sections from two animals. Mean areas in  $\mu\text{m}^2 \pm$  S.D.

NA was identified by inputs from the basilar papillar nerve branch, its rostral position in the neuraxis, and by projections to the superior olivary nucleus and torus semicircularis. The details of the projections of NA will be described in a later paper.

### 3.4 Nucleus magnocellularis

NM was located close to the dorsal surface of the brain, above the medial vestibular nucleus. In transverse sections, NM occupied a smaller area than NA, and in contrast to NA, did not form a distinguishable eminence. CB immunohistochemistry allowed for identification of NM, and serial sections of the CB-ir (CB-immunoreactive) material yielded approximately 40 CB-ir neurons in the location of NM (Figure 2.7b). Since we were unable to identify nucleoli unambiguously in the immunohistochemical material, the NM cell counts are given as an approximation. NM neurons were also lightly positive for PV.

Because of its small size and low cell count, NM was difficult to detect in the Nissl material (Figure 2.7a). We were, however, able to use papillar nerve projections to identify the rostral border of NM as medial and caudal to NA. The boundaries between the two nuclei were clearly demarcated (Figure 2.5a). The caudal border of NM extended slightly caudal to the VIIIth nerve root. The majority of cells in NM were round, with a smaller population of similarly sized fusiform cells. Measurements of cell morphometrics are compiled in Table 2.2. NM neurons were measured at maximum diameter in each 50  $\mu\text{m}$  section since nucleoli were difficult to identify in immunostained material.

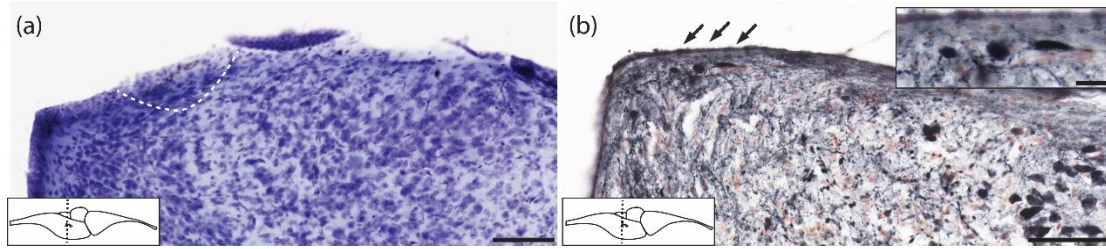


**Figure 2.7 Nucleus magnocellularis in the western ratsnake**

(a) Location of NM (dashed line) corresponding to the area defined in the CB-ir material and nerve tracing results, stained with cresyl violet. Note the lack of distinct nuclear boundaries in this area. Inset: Lateral view showing plane of section. Scale bar = 100  $\mu\text{m}$ .

(b) CB-ir NM neurons (arrows). Scale bar = 100  $\mu\text{m}$ . Inset top right: Magnified view of NM neurons. Note the presence of both round and fusiform cells. Scale bar = 20  $\mu\text{m}$ .

NM was identified by several features: its caudal position in the neuraxis, the receipt of primary afferent input from the basilar papilla, and lack of projections to the superior olivary nucleus and the torus semicircularis. The details of the projections of NM will be described in a later paper.

#### 4. Discussion

Since the studies of Miller (1980) and Defina (1981), both of whom used anterograde axonal degeneration of the VIIIth nerve, there have been no further attempts to examine the auditory brainstem neuroanatomy in snakes. In the current study, we determined the boundaries, size, and potential connections of the first-order cochlear nuclei of the western ratsnake (*Pantherophis obsoletus*), using a combination of Nissl staining, tract tracing, and immunohistochemistry. We have also measured the hearing sensitivity of this species.

Despite the absence of tympanic ears, snakes can hear. Vibration evoked potential recordings demonstrate that the western ratsnake has peak sensitivity in the low frequency range (50-100 Hz), similar to that of other snakes (Christensen et al., 2012; Hartline, 1971b). However, the vibration threshold for the western ratsnake was about 25 dB higher at the low frequencies, and about 10 dB higher at higher frequencies, than the ball python and rattlesnake (Christensen et al., 2012; Hartline, 1971b). This discrepancy may result from differences in methodology. We used isoflurane as the anesthetic agent, while Christensen et al. (2012) and Harline (1971) used ketamine and sodium pentobarbital, respectively. Compared to injection anesthetics, isoflurane has been reported to increase auditory evoked response

thresholds in barn owls and rodents (Cederholm et al., 2012; Ruebhausen et al., 2012; Stronks et al., 2010; Thiele & Köppl, 2018). The sensitivity to aerial sound of western ratsnakes assessed using cochlear microphonics reported best frequencies around 400 Hz (Wever, 1978), higher than that in our findings. This discrepancy may be attributed to their low sample size (n=2).

#### 4.1 The auditory nerve projections are similar to low-frequency fibers in other reptiles

The number of papillar nerve fibers counted in our study (838) was consistent with Miller & Beck (1990), who counted 824 fibers in the same species. Western ratsnake auditory nerve fibers were similar in size to other reptiles. For example, the mean diameter of tectorial and free-standing fibers respectively (likely excluding myelin, preganglionic) was 2.81  $\mu\text{m}$  and 1.97  $\mu\text{m}$  in the alligator lizard (Mulroy & Oblak, 1985), comparable to 2.2  $\mu\text{m}$  of the western ratsnake. A large-scale survey of auditory nerve fibers (including myelin, preganglionic) in 37 lizards reported that the majority of axon diameters fell in the 2-4  $\mu\text{m}$  range (Miller, 1985), which also overlaps that of the western ratsnake (3.3  $\mu\text{m}$ ). Similar measurements of preganglionic fibers were found in birds (Köppl, 1997; Köppl et al., 2000). The g-ratio of axons were also comparable to that of birds (Köppl, 1997; Köppl et al., 2000).

Projections from the western ratsnake basilar papilla entered the medulla where they bifurcate and terminate in two non-overlapping nuclei, NA and NM, following the diapsid pattern (Carr, 2020; Walton et al., 2017). These auditory nerve projections were similar to those of lizards, although studies that measured

postganglionic nerve fiber diameters from tract tracing material reported larger diameters in alligator lizards and geckos (Szpir et al., 1990; Tang et al., 2012). These differences may arise from methodological variation, since Szpir et al. (1990) measured HRP labeled fibers in 50 $\mu$ m thick sections. Tang et al. (2012) found that fibers projecting to NM were thicker than those projecting to NA in the gecko, whereas in the western ratsnake, NM collaterals were thinner than NA collaterals. Moreover, in birds, relatively thicker myelination was observed in smaller axons (Köppl et al., 2000); in contrast, the thin NM collaterals of the western ratsnake had myelination comparable with the larger diameter NA collaterals.

The terminals of the western ratsnake auditory nerve formed round boutons in NM and NA. We found no evidence of the large axosomatic terminals resembling endbulbs of Held that can be found in lizards, birds, alligators and mammals (Carr & Boudreau, 1991; Carr & Soares, 2002; Szpir et al., 1990; Tang et al., 2012). Endbulb synapses are hypothesized to facilitate phase-locking at high frequencies (Carr et al., 2001; Carr & Soares, 2002; Hong & Sanchez, 2018). Their absence was unsurprising, since the low best frequency region of the barn owl NM (< 1 kHz) and the chicken NM (< 500 Hz), which overlaps with the vibration detection range of western ratsnakes, also receives bouton terminals instead of endbulbs (Fukui & Ohmori, 2004; Köppl, 1994; X. Wang et al., 2017). The low frequency region of NM (NM<sub>c</sub>) in birds contains a distinct stellate cell type which receives auditory nerve bouton terminals (Köppl & Carr, 1997; X. Wang et al., 2017), which we also observe in the western ratsnake. Turtles share similar best frequencies with the western ratsnake (Christensen-Dalsgaard et al., 2012; Crawford & Fettiplace, 1980), and also do not

have large terminals in NM (K. L. Willis, 2014), contrary to a prior report (Browner & Marbey, 1988).

#### 4.2 The location of nucleus angularis in the western ratsnake differs from that of lizards

NA forms a dorsal eminence in the acoustic tubercle adjacent to the VIIIth nerve root in the western ratsnake. This corroborates the finding of Weston (1936) in the garter snake (*Thamnophis*), where NA was identified as the only cochlear nucleus. In other studies, it is unclear whether the single auditory nucleus recognized by Holmes (1902), Schwab (1979) and ten Donkelaar & Nieuwenhuys, (1979) was NA. It is possible that a dorsal eminence consisting of only NA does not translate to other snake species (see discussion below on NM). Nevertheless, NA is well-developed in the western ratsnake, and has a similar number of cells to the alligator lizard NA, with 618 neurons (Szpir et al., 1995) versus 580 in the western ratsnake NA.

The western ratsnake NA is located more laterally than NA in lizards. In lizards, NA is separated into medial and lateral divisions, with medial NA located close to the medial surface of the acoustic tubercle (Barbas-Henry & Lohman, 1988; Foster & Hall, 1978; Miller, 1975; Szpir et al., 1990; Tang et al., 2012). The medial division of NA is the only region of the first order nuclei to receive inputs from the specialized high-frequency areas of the basilar papilla with bidirectionally-oriented hair cells (Köppl & Manley, 1992; Manley, 2002; Miller, 1992; Szpir et al., 1990, 1995). The snake basilar papilla, however, does not have specialized high-frequency areas and only houses unidirectional hair cells (Manley, 2002; Miller, 1978, 1980;

Miller & Beck, 1990). Its projections to NA terminate laterally. The most parsimonious explanation for the difference in NA's location is that ancestral snakes secondarily lost the medial high-frequency regions in the squamate basilar papilla, likely related to the loss of their tympanic middle ear (Manley, 2017). NA is also located laterally in archosaurs including birds and crocodilians (Köppl, 2001; Leake, 1974; Soares & Carr, 2001). Considering the early divergence of lepidosaurs and archosaurs (Ezcurra et al., 2014), similarities between the location of the western ratsnake NA and the avian NA may reflect the ancestral pattern, and the medial division of NA in lizards is most likely a derived condition related to the specialized high-frequency region of their basilar papilla.

NA contains heterogeneous cell types; large cells and small round cells were abundant and fusiform cells rare. Similar heterogeneous distributions of cell types characterize the NA divisions in the alligator lizard (Szpir and Ryugo, 1995) and the avian NA (Bloom et al., 2014; Häusler et al., 1999; Soares & Carr, 2001). Projections of first-order cochlear nuclei are not the focal point of this paper (Han and Carr, in preparation), however retrograde label of cells in NA following injections to SO and TS suggests that the ascending projections of NA are similar to that of other reptiles.

#### 4.3 Nucleus magnocellularis is small in the western ratsnake

NM in the western ratsnake is located medially in the acoustic tubercle and caudal to NA, and its location is consistent with observations from previous literature on snakes, and similar to other reptiles (Carr & Boudreau, 1991; Foster & Hall, 1978; Leake, 1974; Miller, 1975, 1980; Molenaar, 1976; Szpir et al., 1990; K. L. Willis &

Carr, 2017). While Miller (1980) described NM as consisting of a nucleus magnocellularis medialis and nucleus magnocellularis lateralis, he also noted that in *Pituophis catenifer* and *Nerodia sipedon* the two divisions were “not clearly separable”, and that NM forms “a cap-like shield over the underlying vestibular nuclei”. In the closely related western ratsnake, we were also unable to differentiate NM into medial and lateral divisions.

The western ratsnake NM is remarkably small, occupying a smaller area than NA in transverse sections, and it does not form a notable dorsal cap. These findings conflict with earlier quantifications of NM and NA in *Pantherophis obsoletus* (formerly *Elaphe obsoleta*; DeFina, 1981; Defina & Kennedy, 1983), where NM nuclear volume ( $0.084 \text{ mm}^3$ ) exceeded NA volume ( $0.022 \text{ mm}^3$ ) in adult animals (average length = 93.1 cm). To compare, in our measurement of an adult animal (case 124, CB-ir, length = 120 cm), the volume of NM was about an order of magnitude smaller than that of NA (NM =  $0.004 \text{ mm}^3$ , NA =  $0.041 \text{ mm}^3$ ). Moreover, Defina & Kennedy’s (1983) average count of 146.2 neurons in NM exceeded our estimate by three-fold. These discrepancies may arise from differences in methodology; quantitative data by DeFina (1981) and Defina & Kennedy (1983) were largely obtained from Nissl-stained material and VIIIth nerve axonal degeneration. During our study, we found that nuclear boundaries of the western ratsnake NM were difficult to delineate solely based on Nissl material (Figure 2.7a). The method of VIIIth nerve degeneration could also lead to unspecific labeling of adjacent vestibular nuclei. While it is possible that there was heterogenous expression of CB in NM, which would lead to our underestimating the number of cells in NM, both the neuron

number and nuclear volume of NM greatly exceeded that of NA in DeFina (1981) and Defina & Kennedy (1983), which suggests that they, and possibly Miller (1980) who used the same methods, may have overestimated the area of NM in some snake species, including the western ratsnake.

The small size of the western ratsnake NM was supported not only by visual comparisons but also by cell counts. The alligator lizard NM has 245 neurons (Szpir et al., 1995), and the avian NM typically exceeds 2000 neurons (Winter, 1963; Winter & Schwartzkopff, 1961), both of which are greater than the approximately 40 neurons found in the western ratsnake. Auditory specialists have higher number of cells in the cochlear nuclei compared to non-specialists (Kubke et al., 2004), and the small number of cells in the western ratsnake NM suggests that the western ratsnake is not an auditory specialist. Furthermore, the presence of a large NA and a small NM suggests that ascending monaural pathways are more prominent than binaural pathways in this snake. We cautiously avoid generalizing the small size of NM to all snake species, since there is evidence for better developed NM in other snakes (personal observations; Miller, 1980; Molenaar, 1976).

#### 4.4 Snakes have a central auditory system for processing vibration despite loss of the tympanic middle ear

Although earlier authors have proposed that snakes diverged prior to the appearance of the tympanic middle ear in the lepidosaur lineage, and that the atympanic middle ear in snakes is ancestral (Miller, 1968; Tumarkin, 1955), more recent paleontological evidence, including that from many crown-group lepidosaur

fossils, reveals an expanded lateral quadrate concavity (conch) that preceded the divergence of snakes (Evans, 2016; Ford et al., 2021; Simões et al., 2018). These findings support the alternative hypothesis, that snakes secondarily lost their tympanic middle ear. Our results suggest that first-order cochlear nuclei remained present despite the loss of an impedance-matching middle ear and tympanum to improve the detection of airborne sound.

Sensitivity of snakes to airborne sound can be explained by sound-induced head vibrations alone (Christensen et al., 2012), which suggests that sound pressure and substrate vibration are both presented to snakes as vibrational stimuli. Therefore, it is reasonable to conclude that snakes, at least to some extent, detect vibrations using the auditory endorgan, the basilar papilla, and centrally process vibrational stimuli in the ascending auditory pathways. While seismic or aquatic vibration are primarily detected by otolithic endorgans such as the saccule in fish and amphibians (Christensen-Dalsgaard & Buhl Jørgensen, 1988; Christensen-Dalsgaard & Carr, 2008; Popper & Fay, 1999), non-otolithic endorgans such as the organ of Corti in mammals and the amphibian papilla in amphibians also facilitate vibration sensing (Capshaw et al., 2022; Christensen-Dalsgaard & Narins, 1993; Stenfelt, 2013; Yu et al., 1991). In snakes, it has been hypothesized that vibrational stimuli impinging on the lower jaw or quadrate may cause movement of the stapes relative to the oval window, causing fluid displacement in the inner ear and stimulation of the basilar papilla (Friedel et al., 2008; Wever, 1978). Currently, parsimony supports the contribution of auditory pathways to vibration detection; *in vivo* recordings from the papillar branch of the VIIIth nerve or cochlear nuclei would provide direct evidence.

Additionally, we do not rule out the possibility that the saccule, or other otolithic endorgans in the inner ear, may also contribute to the snake's sensitivity to vibration.

Given that the basilar papilla may have evolved only once (see Fritzsche et al., 2013 for review), its appearance would have preceded the evolution of the tympanic middle ear, and there likely existed a period in evolutionary history where tetrapods were atympanic but had basilar papillae. Many early tetrapods, such as *Captorhinus*, possessed stapes coupled to the quadrate bone (and indirectly the lower jaw) similar to the condition in snakes (Clack, 1997; Clack & Anderson, 2016; Sobral et al., 2016). While the ability of a snake-like middle ear for detecting airborne sound was likely poor (Christensen et al., 2012; Hartline, 1971; Hartline and Campbell, 1969), our study suggests that it could potentially stimulate papillar pathways to mediate hearing vibrations in early land vertebrates.

## 5. Acknowledgements

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## Chapter 3: Auditory pathway for detection of vibration in the tokay gecko (*Gekko gecko*)

This chapter is a draft of Han, D., & Carr, C. E. in preparation for submission to a journal.

### 1. Introduction

Otolithic endorgans in the vertebrate ear include the saccule, utricle and lagena. These endorgans are made up of a macula or patch of sensory hair cells overtopped by calcareous otoliths or otoconia. The dense otoliths act as an inertial mass that stimulates the sensory hair cells during linear acceleration from gravitational force or movements, providing vertebrates with the vestibular sense. Additionally, in fishes, oscillatory waves produced by underwater sound stimulates the otolithic endorgans by way of either direct particle motion, or indirect sound pressure-induced pulsations of gas-filled chambers such as the swim bladder (Popper et al., 2008). The saccule is the primary auditory end-organ in most teleosts, supported by its responsiveness to sound (Edds-Walton et al., 1999; Fay & Edds-Walton, 1997b; Popper & Lu, 2000) and its ascending projections (Edds-Walton, 1998; Edds-Walton et al., 1999; McCormick, 1999). These ascending projections follows the bauplan for vertebrate auditory pathways (Grothe et al., 2004; McCormick, 1999). In amphibians, the basilar papilla and amphibian papilla are primary recipients of airborne sound, and the saccule remains sensitive to low frequency vibration (Christensen-Dalsgaard & Buhl Jørgensen, 1988; Christensen-

Dalsgaard & Narins, 1993; Christensen-Dalsgaard & Walkowiak, 1999; Jørgensen & Christensen-Dalsgaard, 1991; Koyama et al., 1982; Moffat & Capranica, 1976; Narins & Lewis, 1984; Yu et al., 1991), as a result of relative movement between the sensory epithelia and otoconia caused by traveling waves in the substrate (Lewis & Lombard, 1988; Narins, 1990). Similar to teleosts, saccular projections terminate in the amphibian auditory brainstem (Matesz, 1979; McCormick, 1999; Will et al., 1985a, 1985b; Will & Fritsch, 1988). In archosaurs and mammals, while otolithic endorgans remain naturally sensitive to low frequency vibrations (Curthoys, 2017; Curthoys et al., 2016, 2019; McCue & Guinan, 1994, 1995; E. D. Young et al., 1977), ambiguity exists in their association with auditory pathways (Burian et al., 1989, 1991; Burian & Gstoettner, 1988; Dickman & Fang, 1996; Didier et al., 1987; Gstoettner et al., 1991; A. Kaiser & Manley, 1996; Kevetter & Perachio, 1986, 1989; Newlands et al., 2003; Yingcharoen et al., 2003). The existence of non-cochlear hearing in amniotes remains only speculative (Tait, 1932; Todd et al., 2014; Zeyl et al., 2020).

No studies have investigated whether lepidosaurs, which include snakes, lizards and *Sphenodon*, detect substrate vibration with the saccule or other otolithic endorgans. In this study, we used tract tracing and *in vivo* electrophysiology to investigate contributions of the saccule to auditory processing in the tokay gecko (*Gekko gecko*), which has well-characterized ascending cochlear pathways (Christensen-Dalsgaard et al., 2011, 2021; Eatock & Manley, 1981; Köppl & Authier, 1995; Manley, 1972; Manley et al., 1999; Sammaritano-Klein & Manley, 1976; Sams-Dodd & Capranica, 1994; Tang et al., 2012; Yan et al., 2010). We have

identified a first-order target of the saccule in the acoustic tubercle that projects to the central nucleus of the torus semicircularis and is sensitive to low frequency vibration, suggesting that encoding of vibration in auditory pathways may be more conserved than previously understood.

## 2. Material and Methods

A total of 30 tokay geckos (*Gekko gecko*, snout-vent length  $14.30 \pm 2.05$  cm, weight  $48.89 \pm 20.91$  g,  $n=24$ ) were obtained commercially for this study. Geckos were kept in a 12:12-hour light-dark cycle with 25-34 °C temperature range and offered crickets and water ad libitum. Anesthesia and animal handling protocols were approved by the University of Maryland College Park Animal Care and Use Committee.

### 2.1 Tract tracing and histology

20 geckos were used for tract tracing. Geckos were anesthetized with isoflurane (5%) until fully anesthetized (determined by loss of muscle tone, lack of righting reflex, and lack of reflex to tail pinch). To label afferents from the saccule, the gecko was perfused transcardially with oxygenated ice-cold Ringer's solution [96.5mM NaCl, 31.5mM NaHCO<sub>3</sub>, 4mM CaCl<sub>2</sub>, 2.6mM KCl, 2mM MgCl<sub>2</sub>, and 20mM D-glucose, from Kohl et al. (2014)], then rapidly decapitated and its head placed in a Sylgard-lined dish containing ice cold Ringer's solution. The otic capsule was opened dorsally to expose the membranous labyrinth. The otoconia in the saccule and excessive fluids were removed to expose the saccular macula. Biotinylated

dextran amine (BDA; Dextran, Biotin, 3k MW, Lysine Fixable, Invitrogen, Carlsbad, CA, USA) or tetramethylrhodamine conjugated dextran amine (TMR-DA; Dextran, Tetramethylrhodamine, 3k MW, Anionic, Lysine Fixable, Invitrogen, Carlsbad, CA, USA) crystals were applied to the saccular nerve bundle with a tungsten electrode. The isolated head was placed in a humid chamber in an 8-10 °C refrigerator to allow tracer uptake for 15 minutes, and then rinsed multiple times with Ringer's solution to remove excess tracer. Using the same technique, we labelled the anterior branch of the VIIIth nerve, the nerve bundle from the posterior semicircular canal and papilla neglecta, the basilar papilla and the lagena. The isolated head was immersed in oxygenated Ringer's solution in an 8-10 °C refrigerator for 24-48 hours, with oxygenated Ringer's solution changed every 12-24 hours, to enable tracer transport. The brain was immersion fixed in 4% paraformaldehyde (PFA) in 0.01M phosphate buffered saline (PBS) for 24 h, followed by 24 h cryoprotection in 30% sucrose in 0.01M PBS. Coronal brain sections were cut on a freezing microtome at 50 µm. The membranous labyrinth was also dissected free to visualize tracer spread and possible contamination into other end-organs. To visualize BDA, free floating sections were washed and incubated in avidin-biotin complex (ABC; Vector Laboratories, Burlingame, CA) and then stained with SG chromogen (Vector Laboratories). Sections were mounted on gelatin coated slides, air dried and counterstained with Neutral Red. The membranous labyrinth was cleared in 90% glycerol and visualized with a light microscope (Olympus BX60). Images were photographed with Neurolucida (MBF Bioscience, Williston, VT, USA) in conjunction with a light microscope (Olympus BX60). To visualize TMR-DA, sections and the membranous

labyrinth were mounted in Vectashield/DAPI (Vector Laboratories). Images of brain sections were acquired using a Zeiss LSM 980 Airyscan 2 confocal laser scanner microscope (CLSM, Zeiss, Oberkochen, Germany). Z-stack images of the membranous labyrinth were acquired using a Leica Stellaris 8 FALCON microscope (Leica, Mannheim, Germany). We found that a hindbrain nucleus, nucleus vestibularis ovalis (VeO), exclusively receives input from the saccule.

We injected 10% BDA (in 0.01M PBS; equal parts pf 3k MW and 10k MW) iontophoretically into VeO using an alternating positive current of 2  $\mu$ A for 10-30 minutes to determine the ascending projections of VeO. We also iontophoresed 3% Neurobiotin (NB; Vector Laboratories, Burlingame, CA) in 0.3M NaCl using an alternating positive current of 2  $\mu$ A for 10-20 minutes into the central nucleus of the torus semicircularis (TSC) to determine whether TSC receives input from VeO. Tracer injections into VeO or TSC were made *in vivo* (see below). Geckos recovered for 48 h in their home cages before they were euthanized and perfused with 4% PFA. The brain was extracted and processed as described above.

One additional gecko was processed for Nissl staining to determine the cytoarchitecture of VeO. After fixation, cryoprotection, sectioning and mounting the brain as described above, slides were then stained with cresyl violet, coverslipped, and imaged. To examine and compare VeO in other lepidosaur species, we imaged Nissl-stained transverse sections of VeO in a tuatara (*Sphenodon punctatus*), a green anole (*Anolis carolinensis*), and a western ratsnake (*Pantherophis obsoletus*).

## 2.2 Electrophysiology

In vivo recordings from 14 geckos (including six geckos used for tract tracing) were used to investigate the response properties of VeO. Geckos were anesthetized in an induction chamber with 5% anesthesia, then intubated. A constant flow of carbogen mixed with 1-3% isoflurane at 2-4 ml/min was maintained via a tube into the trachea, and respiration continued under these conditions. The cerebellum and the central portion of the eighth nerve were exposed via a dorsal surgical approach. The choroid plexus and vertebral artery were carefully moved aside to access VeO, which is located medial to the root of the posterior branch of VIIIth nerve. We glued a stainless-steel head post to the prefrontal bone to hold the head in a constant position on a custom-made platform.

Pure tone vibration stimuli (10-1000 Hz) were produced with a vibration exciter (Type 4810; Brüel & Kjær, Nærum, Denmark) connected to the platform. Tonal stimuli had a duration of 600 ms, including 100 ms cosine onset and offset ramps. The length of the ramp was at least one cycle of the lowest frequency (10 Hz) to avoid signal distortion. The interstimulus interval was 400 ms. The vibration exciter was calibrated using a piezoelectric accelerometer (Type 4381; Brüel & Kjær, Nærum, Denmark) placed on the platform next to the gecko's head. The accelerometer was connected to a conditioning amplifier (Type 1704; Brüel & Kjær). The accelerometer itself was calibrated using a calibration exciter (Type 4294; Brüel & Kjær) at  $10 \text{ ms}^{-2}$ , 159.15 Hz with fixed attenuation to levels around -13 dB re  $1 \text{ ms}^{-2}$ . Output of vibration exciter at all stimulus levels were measured in two geckos with the accelerometer to check calibration. Pure-tone vibration stimuli (10 Hz- 1000 Hz)

was generated using custom-written MATLAB software (*tytology*). Signals were fed through a processing device (RX8; Tucker-Davis Technologies, Alachua, FL) and a programmable attenuator (PA5; Tucker-Davis Technologies). Stimulus levels ranged from -53 dB re 1 ms<sup>-2</sup> to -13 dB re 1 ms<sup>-2</sup>.

Recordings were obtained with tungsten microelectrodes (F. Haer, Bowdoin, ME) with impedances around 20 MΩ. Location of VeO was estimated based on histological sections. Electrodes were positioned medial to the posterior branch of the eighth nerve and advanced in 5-10 μm steps by a micro-drive system (μD-800; Walsh) while continuously testing for responses to vibration using different pure tone stimuli. Recorded extracellular signals were amplified (μA-801D; Walsh Electronics) and bandpass-filtered (300-12000Hz), passed through a Hum Bug (Quest Scientific, North Vancouver, BC) to eliminate electrical interference, and converted from analog to digital at 48.8 kHz (RX8). Recordings were visualized online with *tytology* (Go Ashida, Sharad Shanbhag). We measured rate-intensity functions with co-varying frequency and level, with 5 or 10 repetitions per stimulus. Tones of different frequencies and levels were presented in random order. We limited the number of repetitions to minimize electrode drift. Each locus also included multiple (> 40 repetitions) duration-matched recordings of spontaneous activity.

Background noise on the shaker platform caused by environmental vibrations and electrical noise from the amplifier was measured for all stimulus frequencies. To measure noise, we made 80 recordings with a time window of 600ms corresponding to the duration of one stimulus. Each recording was windowed by a Tukey window ( $\alpha=0.1$ ) and filtered by a third-order Butterworth filter with cutoff frequencies

corresponding to one octave band around center frequency. The noise level was then calculated as r.m.s. noise level for the filtered 600 ms recording.

To determine whether responses in VeO were facilitated by the sound emission from the vibration exciter, we measured sound levels (20 Hz-300 Hz) during vibration presentation with a sound level meter (Type 2250; Brüel & Kjær) a half inch microphone (Type 4189; Brüel & Kjær) placed at the level of the gecko's tympanum. Then, in one animal (GG30), we delivered ipsilateral sound stimuli of 20 Hz at 60 dB SPL and 30-300 Hz at 65 dB SPL (at least 8 dB above sound emission levels from the vibration exciter) at frequencies in the 20 to 300 Hz range to vibration sensitive VeO units ( $n = 2$ ). There were no responses to the sound stimuli. Sound stimuli was delivered by an earbud loudspeaker (Yuin PK2) pressed on the earlids and sealed with Gold Velvet ear impression material (All American). The earbud was calibrated using microphones (Knowles EM 3068) close to the eardrum, and the microphone was calibrated using a 1/2-inch reference microphone (Type 4165; Brüel & Kjær). Pure tone sound stimuli (20 Hz to 1000 Hz) were generated using *tytology*, and fed to a processing device (RX8, Tucker Davis Technology), attenuators (PA5, Tucker Davis Technology), and headphone buffers (HB7, Tucker Davis Technology). Sound stimuli time parameters matched that of vibration stimuli described above.

Signal was reprocessed offline using a combination of custom-written MATLAB software and UltraMegaSort2000 (D. N. Hill et al., 2011). Putative spikes were detected by threshold of the voltage trace of all recordings done at the same location at 2.3 times the standard deviation with a 0.6 ms shadow period. Events were then overclustered using k-means and merged based on interface energy between

clusters (D. N. Hill et al., 2011). Putative single units were manually inspected based on the general spike waveform, estimated number of subthreshold spikes, inter-spike interval refractory period violations and autocorrelation function. 38 out of 65 recordings contained one or two well-isolated single units, and the remaining were counted as multi-unit recordings.

We used custom written MATLAB software to analyze spike data. Threshold levels for each frequency were determined as 2 spikes/s above the estimated spontaneous rate and increased in firing rate in the next stimulus level (+5 dB). Best frequency (BF) was determined as the frequency with the lowest threshold. We measured phase-locking using a period histogram, where the instantaneous firing rate is plotted as a function of stimulus phase in cycle. Degree of phase-locking was quantified using vector strength (Goldberg & Brown, 1969). Interspike interval histograms and peristimulus time histograms for all spikes during the stimulus were calculated and plotted. Firing rate was normalized against highest firing rate in rate-level curve and iso-intensity frequency rate curve plots.

### 3. Results

3.1 Nucleus vestibularis ovalis (VeO) receives input from the saccule and projects to the auditory midbrain

The VIIIth nerve includes an anterior branch that receives input from the anterior and lateral semicircular canals and utricle, and a posterior branch that receives input from the posterior semicircular canal, saccule, basilar papilla, and lagena (Baird, 1970; Wever, 1978). To determine the first-order targets of these

different inner ear end-organs, we placed crystallized biotinylated dextran-amine (BDA) or tetramethylrhodamine conjugated dextran-amine (TMR-DA) on the anterior branch of the VIIIth nerve, as well as the individual nerve bundles supplying the posterior semicircular canal, saccule, and basilar papilla and lagena. We identified a first-order nucleus in the acoustic tubercle of the hindbrain, nucleus vestibularis ovalis (VeO), that received input from the saccule (Figure 3.1). VeO has a spherical profile and is located adjacent to the nerve root of the posterior branch of the VIIIth nerve (Figure 3.1A, B). It lies lateral to nucleus magnocellularis (NM) and dorsal to nucleus vestibularis ventrolateralis (Vevl). VeO does not receive input from other inner ear endorgans.

To determine whether VeO is a part of the auditory pathway, we made iontophoretic injections of neurobiotin (NB) in the auditory midbrain of the tokay gecko, the central nucleus of the torus semicircularis (TSC). Neurons in VeO were retrogradely labelled (Figure 3.2A). In transverse sections, VeO neurons had a round or ellipsoid profile, with multiple primary dendrites (Figure 3.2B). We also injected BDA in VeO and anterogradely labelled terminals and axons in TSC (Figure 3.2C, 3.2D). Projections from VeO to TSC were mainly contralateral. Fibers from VeO entered TSC laterally and ventrally, and produced densely labeled neuropil in the caudalmost TSC (Figure 3.2D). In more rostral sections, sparsely labelled axons followed white matter tracts between TSC and the superficial nucleus of the torus semicircularis (TSS) and terminated in the dorsal part of TSC.

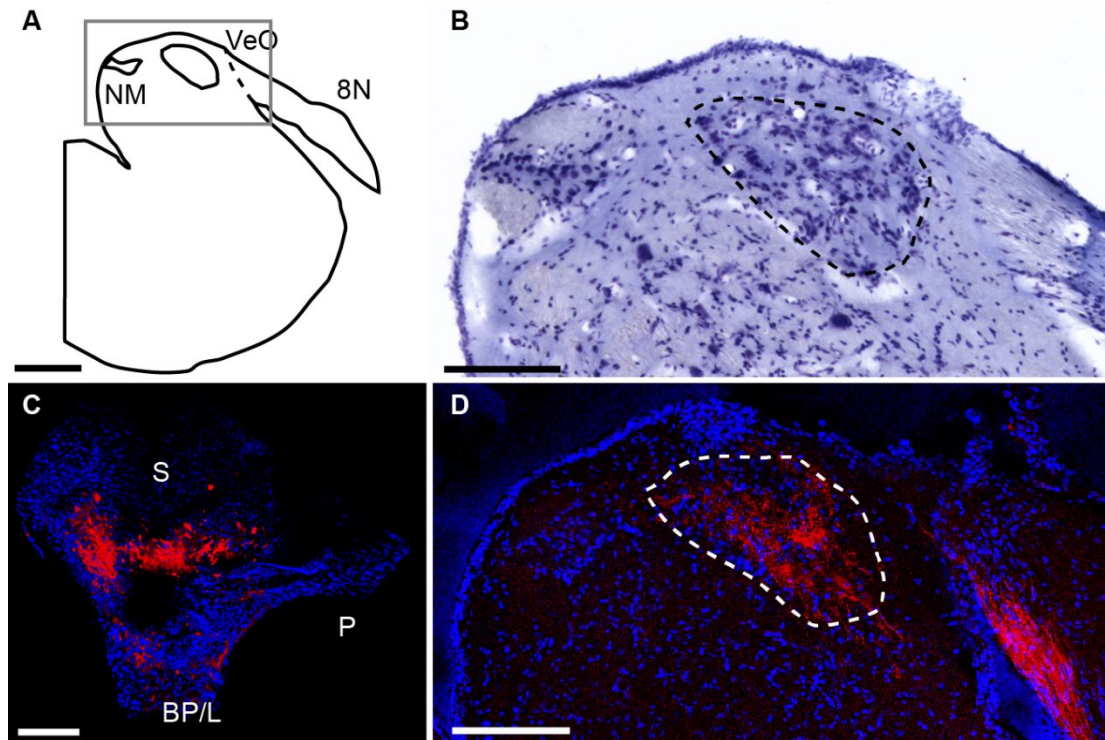


**Figure 3.1 Nucleus vestibularis ovalis (VeO) receives input from the saccule.**

A. Diagram of transverse view of the location of VeO. NM, nucleus magnocellularis; 8N, eighth cranial nerve.

B. Matching microphotograph of a Nissl-stained section showing VeO (dashed line).

C. Reconstructed confocal Z-stack showing cells and nerve fibers labelled by TMR-DA in the saccular branch of the VIIIth nerve following dye placement in the saccule. Note few fibers were labelled in the branch innervating posterior semicircular canal (P) or the basilar papilla and lagena (BP/L). Counterstained with DAPI.

D. Labelled terminals and fibers in VeO (dashed line) and labeled saccule nerve fibers following TMR-DA crystal placement in the saccule. C and D were from the same animal.

Scale bars = 500  $\mu\text{m}$  in A; 200  $\mu\text{m}$  in B-D.

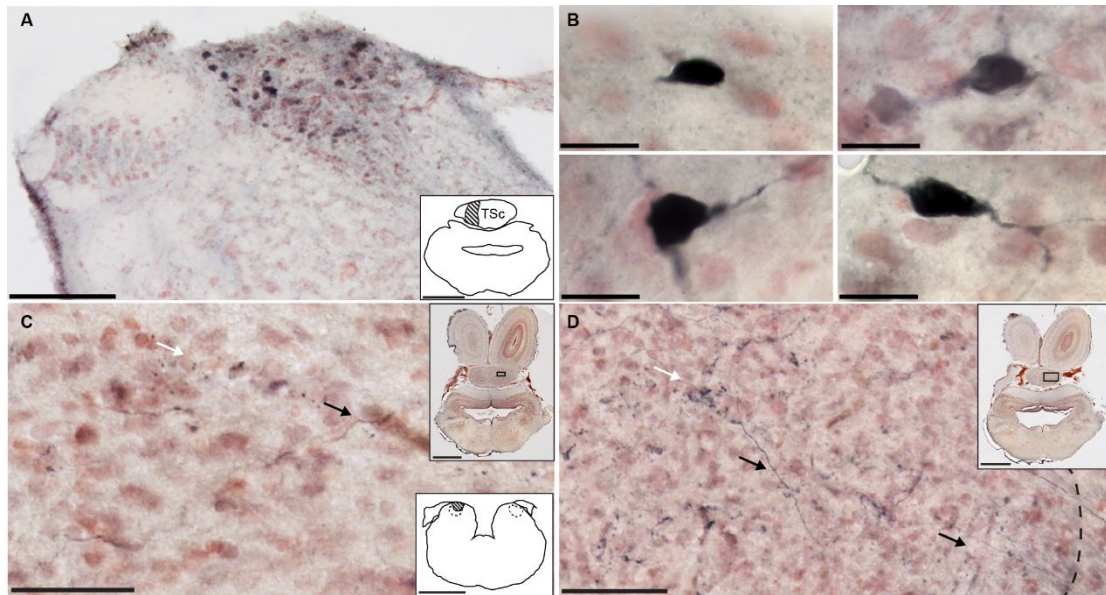


**Figure 3.2 VeO projects to the central nucleus of the torus semicircularis (TSc)**

A. Retrogradely labelled neurons in VeO following injection in TSc. Inset: injection site in TSc (diagonal lines).

B. Labelled neurons in VeO are multipolar with round or ellipsoid cell bodies.

C. Sparsely labelled terminals (white arrow) and fibers (black arrow) in the dorsal part of TSc following injection of biotinylated dextran-amine (BDA) into VeO. Inset top location of image in the transverse brain section. Inset bottom injection site (diagonal lines) in VeO (dotted lines).

D. Extensively labelled terminals (white arrow) and fibers (black arrow) in more caudal sections of TSc (left of dashed line). Projections from VeO enter from the ventrolateral direction (bottom right black arrow). Injection site is the same as C. Inset location of image in the transverse brain section.

Scale bars = 200  $\mu\text{m}$  in A; 20  $\mu\text{m}$  in B; 50  $\mu\text{m}$  in C; 100  $\mu\text{m}$  in D; 1 mm in insets.

VeO also projected to the superior olivary nuclei (SO) and the dorsal nucleus of the lateral lemniscus (LLD; Figure 3.3). Injections in VeO labelled terminals and fibers in the ventral superior olive (SOv), that were more prominent on the contralateral (Figure 3.3A) than ipsilateral side (Figure 3.3B). Sparsely distributed terminals were also found in the ipsilateral dorsal superior olive (SOd). Both SOv and SOd projected back to VeO. We found retrogradely labelled neurons in the ipsilateral SOv and SOd (Figure 3.3B, 3.3C). In more rostral planes, terminals and fibers were labelled in the posterior (LLDp) and anterior (LLDa) division of the dorsal nucleus of the lateral lemniscus, mainly on the contralateral side (Figure 3.3D, 3.3E).

In summary, VeO received input from the saccule and mainly innervated the contralateral SOv, LLDp, LLDa and TSc, with minor projections to the ipsilateral SOd, SOv, LLD and TSc. The ipsilateral SOd and SOv projected back to VeO (Figure 3.3F). Connections of VeO largely mirrored those of cochlear nuclei (Tang et al., 2012). Contrary to earlier reports (Song et al., 2013), injections in VeO did not label the oculomotor nucleus.

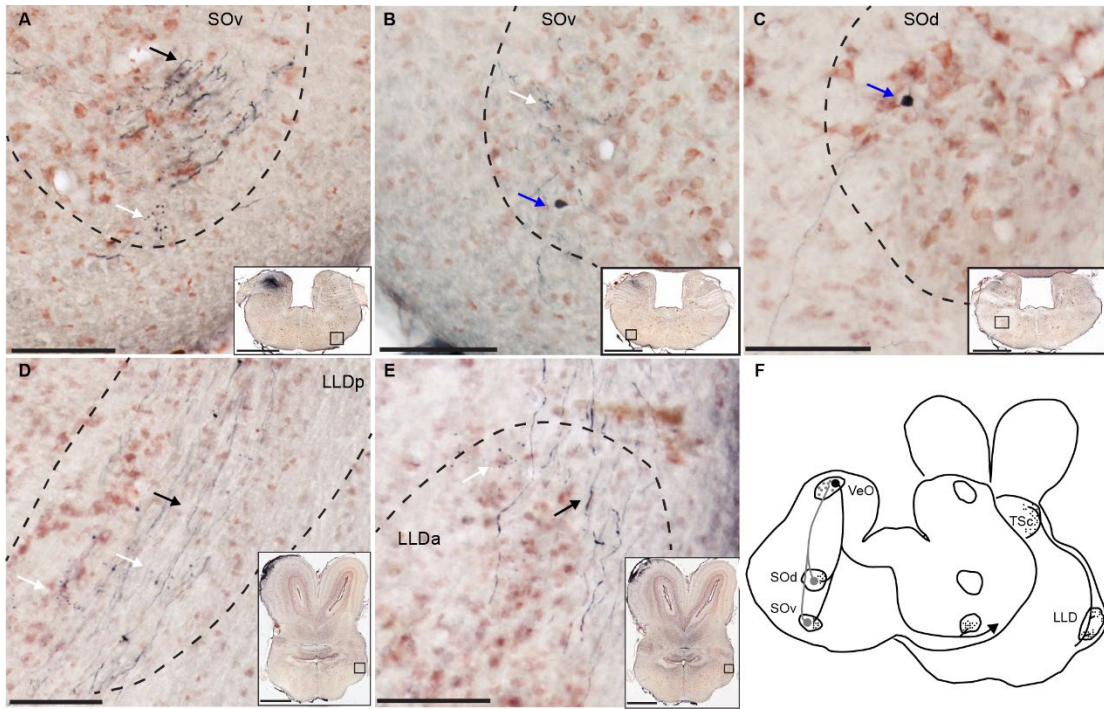


**Figure 3.3 Connections of VeO mirror first-order cochlear nuclei**

A. Labelled fibers (black arrow) and terminals (white arrows) in the contralateral ventral nucleus of the superior olive (SOv; dashed line) following injection in VeO.

B. Retrogradely labelled neuron (blue arrow) and anterogradely labelled terminals (white arrow) in the ipsilateral SOv (dashed line).

C. Retrogradely labelled neuron (blue arrow) in the ipsilateral dorsal nucleus of the superior olive (SOd; dashed line).

D. Labelled terminals (white arrows) and fibers (black arrow) in the posterior division of the dorsal nucleus of the lateral lemniscus (LLDp; dashed line).

E. Labelled terminals (white arrow) and fibers (black arrow) in the anterior division of the dorsal nucleus of the lateral lemniscus (LLDa; dashed line).

F. Summary of connections of VeO. VeO sends major projections to the contralateral SOv, LLDp, LLDa and TSc and minor projections to the ipsilateral SOd and SOv (black line). SOd and SOv both send reciprocal projections to the ipsilateral VeO (gray line). Connections of VeO are similar to that of cochlear nuclei in the tokay gecko (Tang 2012; Han and Carr, in preparation 2024)

Insets A-E: location of image in transverse brain sections.

Scale bars = 100  $\mu$ m in A-E; 1 mm in insets.

### 3.2 VeO is sensitive to low-frequency vibrations

Considering that VeO received input from the saccule, we hypothesized that VeO was sensitive to low frequency vibration. To test this hypothesis, we characterized the response properties of VeO units using extracellular electrophysiological recordings (Figure 3.4A). We used pure tone vibration stimuli from 10 Hz to 1000 Hz, with duration of 600 ms, including 100 ms cosine onset and offset ramps. The length of the ramp was at least one cycle of the lowest frequency (10 Hz) to reduce signal distortion (Figure 3.4B). Due to the length of the ramp, we were unable to reliably quantify the latency of spikes to stimulus onset. We report the frequency tuning, phase-locking and responses to increasing vibration intensity of 38 single VeO units from 9 animals. Our tungsten electrodes were capable of recording both cell bodies and large axons or dendrites (Joris, 1998; Köppl & Carr, 2003). Since the saccular nerve terminates extensively in VeO, and there are no studies characterizing response properties of the saccular nerve in lizards, we were unable to differentiate whether responses recorded in VeO originated from the saccular nerve or from VeO neurons. VeO units were sensitive to low frequency vibration (Figure 3.4C), and best frequencies (BF) ranged from 50 to 200 Hz (Figure 3.5A). Mean vibration thresholds measured at BF was  $-42.61 \pm 7.20$  dB re  $1 \text{ ms}^{-2}$  (n=38). The lowest single unit thresholds were -53 dB for 3 units with best frequencies of 50 Hz, 100 Hz, and 100 Hz. The highest single unit thresholds were -23 dB for two units with best frequencies of 80 Hz and 200 Hz.

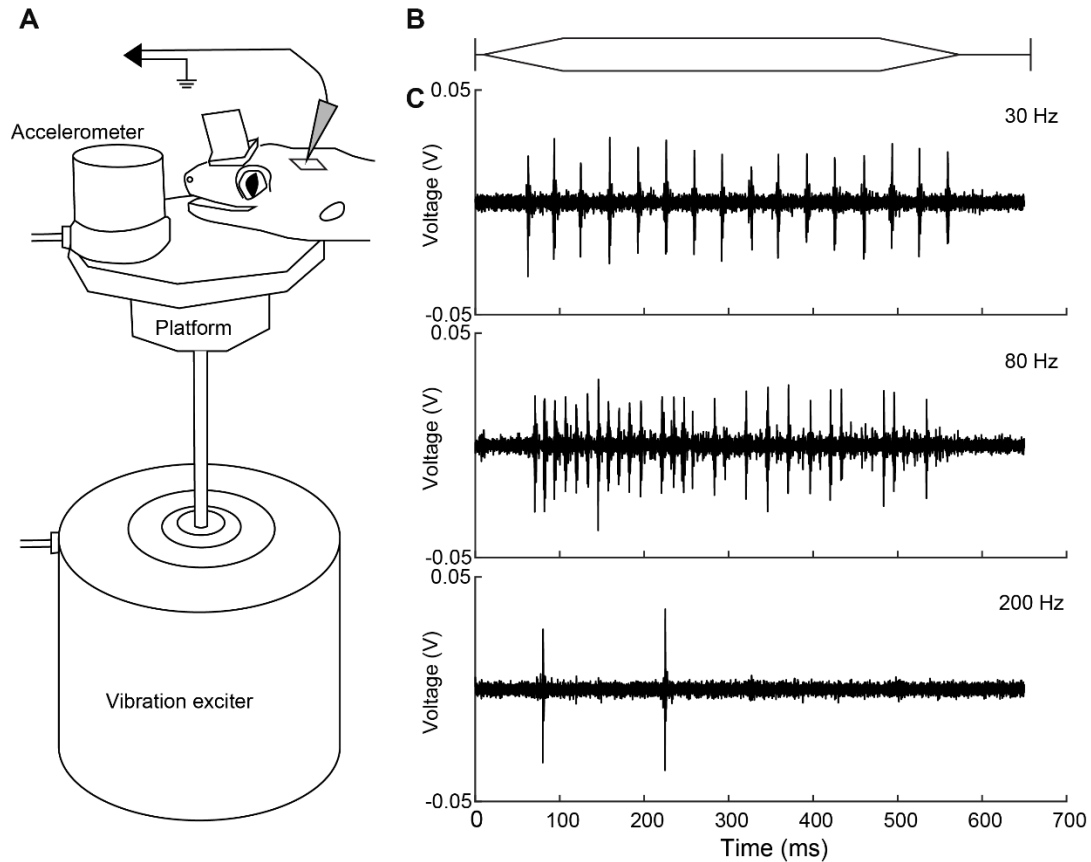


**Figure 3.4 Recordings in VeO in response to vibration**

A. Recording setup. The gecko's head rested on a custom-made platform connected to the vibration exciter. The accelerometer was fixed to the platform with wax, and the vibration exciter was calibrated with the gecko's head in place to account for load.

B. Schematic of time parameters of the vibrational stimuli, including 400 ms of pure tone stimuli and 100 ms on- and off-ramps.

C. Extracellular recording of an example single unit in VeO in response to -18 dB re  $1 \text{ ms}^{-2}$  vibration at 30 Hz (top), 80 Hz (middle) and 200 Hz (bottom).

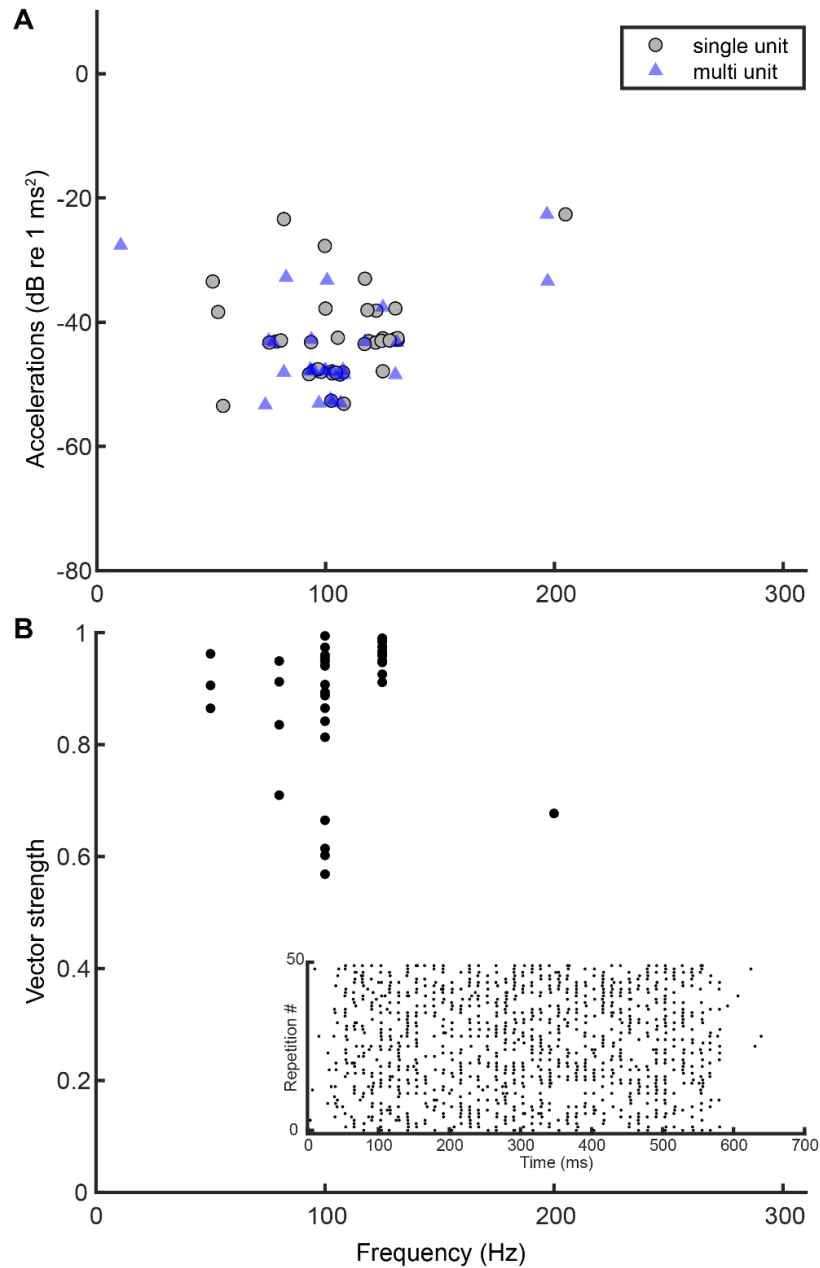


**Figure 3.5 Physiological properties of VeO neurons**

A. Vibration thresholds measured at BF in VeO, with data from 38 single units and 27 multi-units.

B. Vector strength (VS) values for single units measured at BF at 20 dB above threshold (n=35) or 10 dB above threshold (n=3). Inset: Exemplar dot raster plot of a single VeO unit in response to 50 repetitions of BF stimulus (80 Hz) at -23 dB (20 dB above threshold). VS = 0.86.

In our analysis of frequency tuning, we include data from an additional 27 multi-unit recordings where we were unable to isolate single units based on spike sorting criteria. BFs ranged from 80 to 200 Hz, and mean vibration thresholds measured at BF were  $-44.30 \pm 8.04$  dB re  $1 \text{ ms}^{-2}$  ( $n=27$ ). The lowest multi-unit thresholds were -53 dB for five units with best frequencies of 80 Hz, 100 Hz, 100 Hz, 100 Hz, and 100 Hz. The highest multiunit threshold was -23 dB for one unit with a best frequency of 200 Hz. Only one single unit and one multiunit had best frequencies of 200 Hz; most units were most responsive to frequencies below 200 Hz. Frequency tuning of single units and multi-units were similar. Ambient noise levels in one-octave bands around all tested frequencies were at least 5 dB below threshold.

We used measures of vector strength to quantify phase-locking of VeO units at BF (Figure 3.5B; Goldberg and Brown, 1969; Ashida and Carr, 2010), and visualized phase-locking using phase histograms (Figure 3.6). Vector strength falls between 0 (no phase preference for reference signal) and 1 (all spikes occur in the same phase of the signal). Phase histograms plot instantaneous firing rate as a function of stimulus phase in cycles. Only single units were used to quantify vector strength ( $n=38$ ). We found that VeO units reliably phase-locked to the stimulus (Figure 3.6B). In the dot raster plot of a sample unit (Figure 3.6B inset) presented with 50 repetitions of pure tone stimuli at its BF (80Hz), the occurrence of dots aligned vertically, indicating spikes occurring at the preferred phase of the stimulus. In VeO, vector strength varied from 0.61 to 0.99, with an average of  $0.89 \pm 0.12$  ( $n=38$ ). We plotted the preferred phases of all single units at 100 Hz and found that

the preferred phases for most VeO units were about 180 ° apart. This bimodal distribution is also pronounced in the phase histograms of some multiunit recordings.

The firing responses of VeO units were typically primary-like, with a phasic pattern of discharge at stimulus onset followed by tonic discharge at a lower rate for the remaining stimulus period (Figure 3.6A). Other VeO units had a primary-like firing pattern with a more sustained firing rate (Figure 3.6D). We also recorded units that fired only during the onset of the stimulus, characterized by a single prominent peak in their PSTH at the beginning of the stimulus (Figure 3.6G). Units were reliably phase-locked to their preferred cycle (Figure 3.6B, 3.6E, 3.6H). Interspike intervals measured at BF were 8 ms, 20 ms, and 10 ms, corresponding to the stimulus cycle of 125 Hz, 50 Hz, and 100 Hz, respectively (Figure 3.6C, 3.6F, 3.6G). At low stimulus intensities, units skip one or more stimulus cycles, producing multiple peaks in the interval histogram (Figure 3.6C, 3.6F, 3.6G). When stimulus intensities increased, the spike rate typically increased until it reached one spike per cycle.

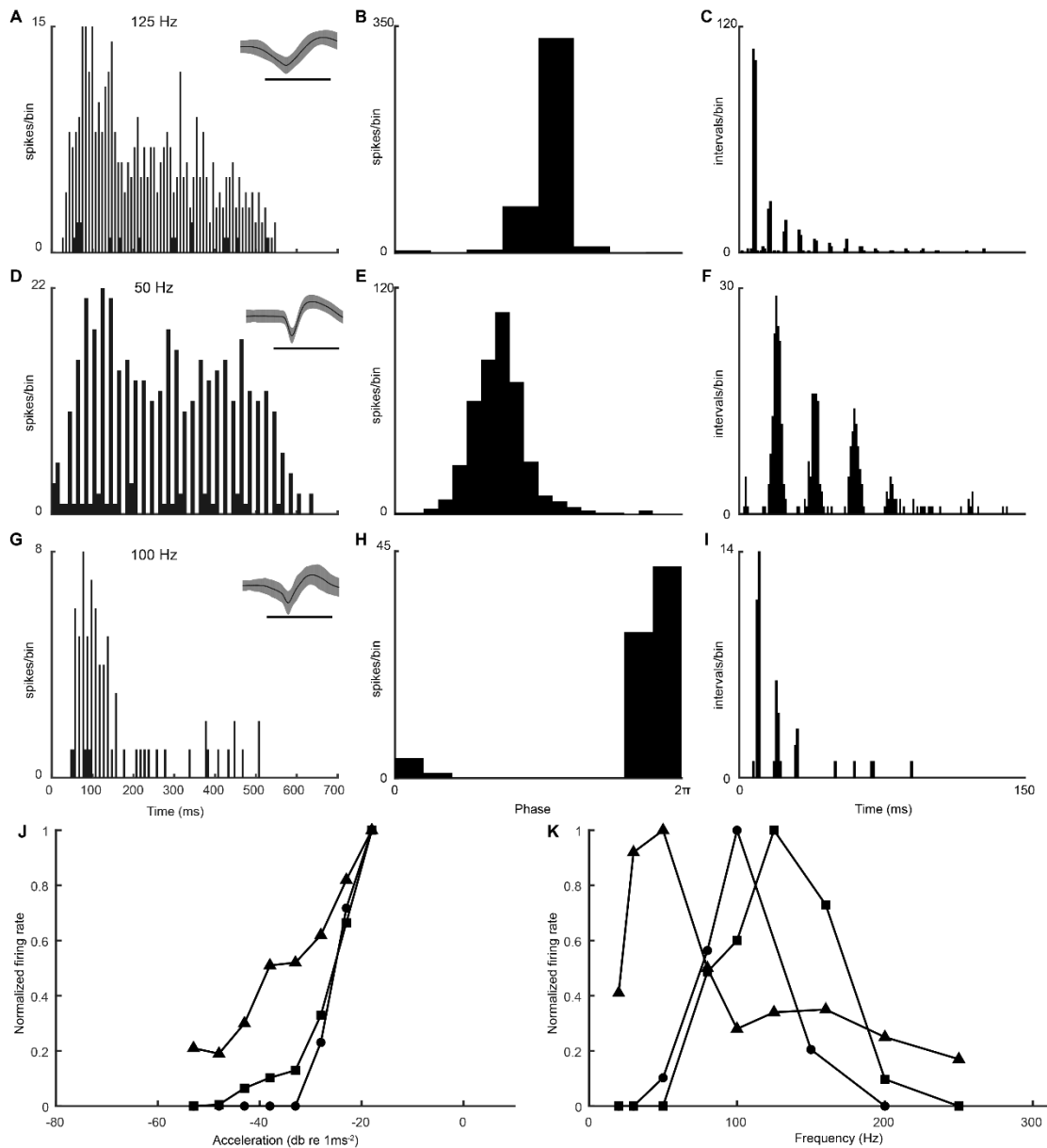


**Figure 3.6 Examples single units in VeO**

A-C Unit with primary-like response (BF = 125 Hz, combining data from -43 dB to -18 dB, 5 repetitions each stimulus level, 30 repetitions total)

A. Peristimulus time histogram (PSTH). Bin width = 4 ms.

B. Period histogram at BF. VS = 0.93. Bin width = 1 ms.

C. Interspike interval histogram. Bin width = 1 ms.

D-F Unit with a primary-like/sustained response (BF = 50 Hz, combining data from -53 dB to -18 dB, 5 repetitions each stimulus level, 40 repetitions total)

D. Peristimulus time histogram (PSTH). Bin width = 10 ms.

E. Period histogram at BF. VS = 0.83. Bin width = 1 ms.

F. Interspike interval histogram. Bin width = 1 ms.

G-I Unit with an onset-type response (BF = 100 Hz, combining data from -28 dB to -18 dB, 5 repetitions each stimulus level, 15 repetitions total)

G. Peristimulus time histogram (PSTH). Bin width = 5 ms.

H. Period histogram at BF. VS = 0.95. Bin width = 1 ms.

I. Interspike interval histogram. Bin width = 1 ms.

J. Rate-level functions of the three example units, measured at BF. Square, unit in A-C; triangle, unit in D-F; circle, unit in G-I.

K. Iso-intensity (-18 dB) frequency-rate curves for the three example units. Square, unit in A-C; triangle, unit in D-F; circle, unit in G-I.

### 3.3 Technical considerations for vibration stimuli

Maximum stimulus levels were limited to -18 dB re  $1 \text{ ms}^{-2}$  for 30 Hz-1000 Hz, and -28 dB for 20 Hz, and -38 dB for 10 Hz, because higher stimulus levels lead to unstable spike waveforms. For example, based on the equation  $\text{displacement} = \text{acceleration} / 4 \pi^2 f^2$ , a vibration stimulus at -18 dB re  $1 \text{ ms}^{-2}$  RMS, or  $0.36 \text{ ms}^{-2}$  peak-to-peak, corresponds to displacement of  $90 \text{ }\mu\text{m}$  peak-to-peak at 10 Hz and  $23 \text{ }\mu\text{m}$  peak-to-peak at 20 Hz. While we recorded responses to 10 Hz and 20 Hz at -18 dB re  $1 \text{ ms}^{-2}$ , the spike waveform varied considerably in height during different cycles of the stimulus period. It seemed likely that the responses were caused by a movement artifact relative to the microelectrode, and these spikes were not included in our analysis. Additionally, at high stimulus levels, recorded waveforms included large extracellular field potentials, or neurophonics, with a prominent peak in amplitude spectrum at the stimulus frequency, with truncated peaks at higher harmonics, similar to those found in cochlear pathways (Kettler & Carr, 2019; Kuokkanen et al., 2018; Snyder & Schreiner, 1984). Bandpass filtering of the recorded extracellular signal at 300 Hz- 12000 Hz eliminated the neurophonic component with amplitude spectrum at stimulus frequency, since units were mostly responsive to vibrations below 300 Hz. However, neurophonic components with amplitude spectrum at higher harmonics of the stimulus frequency (e.g. 375 Hz, 500 Hz for a 125 Hz stimulus) could not be filtered out and affected spike height and shape. The maximum stimulus level was therefore set to -18 dB re  $\text{ms}^{-2}$  for 30 Hz-1000 Hz to limit the effect of the neurophonic on thresholding during spike extraction and spike sorting.

To determine whether VeO responses were possibly caused by sound emission from the vibration exciter, instead of vibration itself, we measured free-field sound from the vibration platform, and then delivered sound stimuli to the animal's ear. At acceleration of -3 dB re 1 ms<sup>-2</sup> (15 dB above the highest vibration level used) the sound levels ranged from 52.4 dB SPL to 59.1 dB SPL at frequencies between 20 Hz and 300 Hz. In three recording sites of one gecko, after delivering vibration stimuli, we also presented pure tone sound stimuli of 20 Hz at 60 dB SPL and 30-300 Hz at 65 dB SPL to the ear ipsilateral to the recording site. No VeO unit responded to the sound stimuli.

For all experiments, the output of the vibration exciter was calibrated to the maximum stimulus levels used in this experiment (-18 dB re 1 ms<sup>-2</sup>). Since the head of the gecko is fixed to a headplate attached to a stereotactic device, the load on the platform theoretically varies during different phases of the stimulus cycle (largest at  $0.5 \pi$ , and smallest at  $1.5 \pi$ ) and at different stimulus levels, potentially leading to inaccurate calibration. To assess the accuracy of the vibration exciter output at different stimulus levels, we calibrated the vibration exciter for 10-1000 Hz at -18 dB re 1 ms<sup>-2</sup> and measured the peak-to-peak output of the accelerometer from -53 dB to -23 dB, with or without stereotaxis (n=3). Error was within 1 dB for all stimuli used. Thus, the effect from the stereotactic headplate was negligible, likely due to flexibility in the tissue and the small displacement of the vibrating platform (90  $\mu$ m peak-to-peak at 10 Hz, less for all other frequencies).

#### 4. Discussion

We have identified an ascending pathway for vibration processing in tokay gecko using tract tracing and in vivo physiology. VeO is a first-order hindbrain nucleus that receives saccular input and terminates in the auditory midbrain, the central nucleus of the torus semicircularis (TSC). Its connections in the brainstem are similar to those of cochlear nuclei, except VeO neurons are sensitive to vibration, with best frequencies between 50 and 200 Hz, and phase-lock to the vibration stimulus. This is the first study to demonstrate vibration processing in auditory pathways in amniotes. The occurrence of separate pathways for detecting vibration and sound suggests that vibration may play a larger role than previously understood in the sensory world of tokay geckos, and possibly all lepidosaurs. The resemblance to ascending projections from the saccule of fishes suggests that vertebrate auditory processing may be more conserved than previously thought.

##### 4.1 The saccule and VeO form part of the ascending auditory pathway in the gecko

The organization of auditory pathways in different vertebrates follows the same basic pattern. The auditory nerve synapses onto first-order octaval nuclei in the dorsal region of the rhombencephalon, which project to higher-order octaval nuclei such as the superior olive and terminate in the auditory midbrain (central nucleus of the inferior colliculus or the torus semicircularis; Grothe et al., 2004; Walton et al., 2017). In land vertebrates such as birds and mammals, which have well characterized auditory systems, airborne sound is detected by the tympanic middle ear, and the

input to the first-order octaval nuclei originates from the basilar papilla or organ of Corti in the cochlea (Fritzsche et al., 2013).

In lizards, connections of brainstem cochlear nuclei have been well-characterized (Barbas-Henry & Lohman, 1988; Foster & Hall, 1978; Szpir et al., 1990; Tang et al., 2012). The cochlear nerve terminates in two first-order cochlear nuclei, nucleus angularis (NA) and nucleus magnocellularis (NM) (Barbas-Henry & Lohman, 1988; Foster & Hall, 1978; Szpir et al., 1990; Tang et al., 2012). NM projects bilaterally to a second-order cochlear nucleus, nucleus laminaris (NL). NL and NA project ventrally to the superior olive (SO), to the nuclei of the lateral lemniscus and the central nucleus of the torus semicircularis (TSC). The lizard SO has a distinct dorsal component (SOd) and ventral component (SOv) (Tang et al., 2012; Foster and Hall, 1978). SOd and SOv both project back to the first-order cochlear nuclei (Tang et al., 2012). The connections of VeO follow the same basic pattern of the vertebrate auditory pathway, mirroring connections of the brainstem cochlear nuclei in the tokay gecko. VeO projects to SO nuclei, and to the posterior and anterior division of the nucleus of the lateral lemniscus. The terminal fields of VeO and NA/NL overlap in the caudal-most TSC as well as the lateral part of the ventral division of TSC (TSCv), and VeO also has a sparse but distinct projection to the dorsal division of the TSC (TSCd).

In teleosts and amphibians, first-order octaval nuclei receive input from otolithic endorgans (McCormick, 1999). The cochlea/papilla is absent in teleost fish; instead, the utricle, saccule and lagena can all serve an auditory function, with the saccule likely playing the major role (Popper & Pay, 1993; Schulz-Mirbach et al.,

2019). The dorsomedial zones of the descending nucleus and, in some species, the anterior nucleus, receive primary auditory input and project to the torus semicircularis (McCormick, 1999). The descending nucleus also sends bilateral projections to a more medially located second-order octaval nucleus (McCormick & Hernandez, 1996; Yamamoto & Ito, 2005). In amphibians, papillar endorgans (the basilar papilla and amphibian papilla) act as sensors of airborne sound pressure, and the saccule retains its “auditory” function as a detector of seismic vibrations (Lewis & Narins, 1999). The primary auditory nucleus is the dorsal medullary nucleus, with the dorsal neuropil receiving input from the papillar endorgans and the ventral neuropil receives input from the saccule (Fuzessery & Feng, 1981; Lewis et al., 1980; Matesz, 1979; Will et al., 1985a, 1985b; Will & Fritzsche, 1988). The dorsal medullary nucleus projects to the superior olivary nucleus, nucleus of the lateral lemniscus and the torus semicircularis (Edwards & Kelley, 2001; Feng, 1986; Wilczynski, 1981; Will et al., 1985b). Saccular recipients in the tokay gecko (VeO), teleosts (descending) and frogs (ventral region of dorsal medullary nucleus) share similar ascending projections, supporting conservation of an ancestral auditory pathway. We note that the left and right descending and dorsal medullary nucleus are connected via a commissural tract in both fishes and frogs (Edds-Walton, 1998; Edds-Walton & Fay, 2009; Feng, 1986; Grofová & Corvaja, 1972; Will et al., 1985b), which we did not observe in VeO.

#### 4.2 The saccule is exquisitely sensitive to vibration in the tokay gecko

In the tokay gecko, VeO exhibits remarkable sensitivity to 50-200 Hz vibrations, with best frequencies around 100 Hz. The range of best frequencies is

similar to saccular fibers of many amphibians. Saccular fibers in the American bullfrog (*Rana catesbeiana*), white-lipped frog (*Leptodactylus albilabris*) and northern leopard frog (*Rana pipiens*) had best frequencies of 20-150 Hz, 20-160 Hz, and 10-80 Hz, respectively, and were band-pass tuned to frequencies up to 300 Hz (Christensen-Dalsgaard & Narins, 1993; Koyama et al., 1982; Narins & Lewis, 1984), comparable to our findings in the tokay gecko VeO. Measurement of inner ear microphonics (Ross & Smith, 1980) and auditory brainstem responses (Capshaw et al., 2020) of salamanders show similar best vibration sensitivity around 100 Hz, likely imparted by the saccule. Lungfish (*Protopterus annectens*) are also sensitive to low frequency vibrations at 40-60 Hz, shown in auditory brainstem response to be mediated by the otolithic endorgans (Christensen et al., 2015). Snakes are responsive to low-frequency substrate vibrations, with best sensitivities of -62 dB to -30 dB at 50-200 Hz, depending on species (Christensen et al., 2012; Han & Carr, 2023; Hartline, 1971b). However, determination of the associated inner ear endorgan is complicated by cochlear pathways, which have been shown to contribute to detection of substrate vibration in snakes (Wever, 1978). This study is the first clear demonstration of an amniote, the tokay gecko, using otolithic endorgans to sense vibrations, with comparable sensitivity to anamniotes.

Units recorded in VeO show clear phase-locking. Similar phase-locking was seen in saccular nerve fibers of fish (Fay, 1978; Fay & Edds-Walton, 1997b, 1997a; Lu et al., 1998) and frogs (Christensen-Dalsgaard & Buhl Jørgensen, 1988; Christensen-Dalsgaard & Narins, 1993; Jørgensen & Christensen-Dalsgaard, 1991; Koyama et al., 1982; Narins & Lewis, 1984). First-order saccular nuclei in teleosts

and frogs also phase-lock to low frequencies (Christensen-Dalsgaard & Walkowiak, 1999; Edds-Walton & Fay, 2008, 2009). There is a bimodal distribution of preferred phases, with most units having preferred phases  $180^\circ$  apart, indicative of oppositely oriented hair cell populations on the saccular macula, a feature shared among vertebrates (Lewis et al., 1985; Sienknecht, 2013). Units with primary-like and phasic firing patterns found in VeO have also been described in the first-order saccular nuclei of fish and frogs (Christensen-Dalsgaard & Walkowiak, 1999; Edds-Walton & Fay, 2008; Page, 1970; Wubbels et al., 1993).

The largest differences between the vibration sensitivity of the tokay gecko VeO and the frog saccule is that frog saccular fibers were more sensitive than VeO and most had higher spontaneous firing rates. Spontaneously firing saccular fibers in bull frogs, white-lipped frogs, and the northern leopard frog, which have overlapping best frequencies with gecko VeO units, were sensitive to accelerations as low as  $0.001\text{-}0.01\text{ cm}^{-2}$ , equivalent to  $-100\text{ - }-80\text{ dB re }1\text{ ms}^{-2}$  (Christensen-Dalsgaard & Narins, 1993; Koyama et al., 1982; Narins & Lewis, 1984; Yu et al., 1991). Non-spontaneous units, which comprised a smaller subset of frog saccular fibers, typically had higher thresholds (Christensen-Dalsgaard & Buhl Jørgensen, 1988; Koyama et al., 1982), responding to vibration levels in the neighborhood of  $0.1\text{ cm s}^{-2}$ , equivalent to  $-60\text{ dB re }1\text{ ms}^{-2}$ , only slightly lower than VeO thresholds (Koyama et al., 1982). The differences between VeO units and frog saccular fibers may be attributed to our use of isoflurane, which is known to depress spontaneous activity and decrease sensitivity in the gecko auditory nerve (Dodd & Capranica, 1992). On the other hand, the lower spontaneous firing rates and the higher thresholds for VeO may reflect

interspecific variation. Sacculus hair cell sensitivity is linked to the number of stereocilia per hair cell (Hudspeth & Jacobs, 1979), and there are little available data on cytoarchitecture of sensory epithelia of otolithic end-organs in non-avian reptiles (Lewis et al., 1985; Sienknecht, 2013).

No behavioral role for a dedicated vibration receptor has been demonstrated in the tokay gecko. The dominant frequency (>695 Hz) of the tokay gecko call is higher than the sensitivity range of VeO (Tang et al., 2001). Vibrational responses are likely associated with sensing the low-frequency component in disturbances from abiotic forces such as wind or rain (Roberts & Howard, 2022; Tsubaki et al., 2014) or during predator-prey interactions (Christensen-Dalsgaard & Buhl Jørgensen, 1988; Han & Young, 2022; Hartline, 1971b; B. A. Young, 2003; B. A. Young et al., 2000).

#### 4.3 Sacculus hearing- a possibility in other species?

VeO is a distinct brainstem nucleus widely found in lepidosaurs. The name VeO was taken from (Foster & Hall, 1978), where it was recognized in the green iguana (*Iguana iguana*) as a recipient of VIIIth nerve input using nerve degeneration. Similarly, Barbas-Henry & Lohman (1988) used HRP to label the posterior root of the VIIIth nerve in *Varanus exanthematicus*, and found terminals in VeO, naming it “caudal cluster”. In earlier literature, Glatt (1975b) found a “nucleus lagenaris” that corresponded to the location and cytoarchitecture of VeO in seven other species of lizards across families Varanidae, Teiidae, Lacertidae, Chamaeleonidae and Gekkonidae. VeO was also depicted in Figure 10 of Weston (1936) in *Holbrookia sp.* and Figure 16 of ten Donkelaar & Nieuwenhuys (1979) in *Tupinambis*

*nigropunctatus* but not discussed in detail. By surveying serial Nissl sections of lepidosaurs, we found VeO in other lizards, snakes, and the tuatara (Figure 3.7). In one snake species, preliminary evidence suggests that VeO receives saccule afferents and projects to the torus (unpublished). The non-cochlear origin of VeO input is also supported in the green iguana (Foster & Hall, 1978). To what extent the function and connections of VeO match the gecko blueprint awaits further experimentation.

In turtles and alligators, we are uncertain whether a nucleus comparable to VeO exists, based on location and cytoarchitecture. The “nucleus lagenaris” described in lizards, i.e. VeO, differs from the “nucleus lagenaris” in turtles and alligators in position, shape and size (Glatt, 1975b, 1975a). Leake (1974) recognized a group of ovoid cells that mainly receives fibers from the saccule and its location seems to overlap, at least partially, with the “nucleus lagenaris” of Glatt (1975a). However, its ascending projections are unclear. Among birds, a study in pigeons shows the saccule projects to vestibular nuclei, which also receive input from other otolithic endorgans, suggesting a vestibular function (Dickman & Fang, 1996). While there are additional regions near the cochlear nuclei with toral projections found in chicks, collectively referred to as *regio intermedius* (Y. Wang & Karten, 2010), their input is yet to be determined.

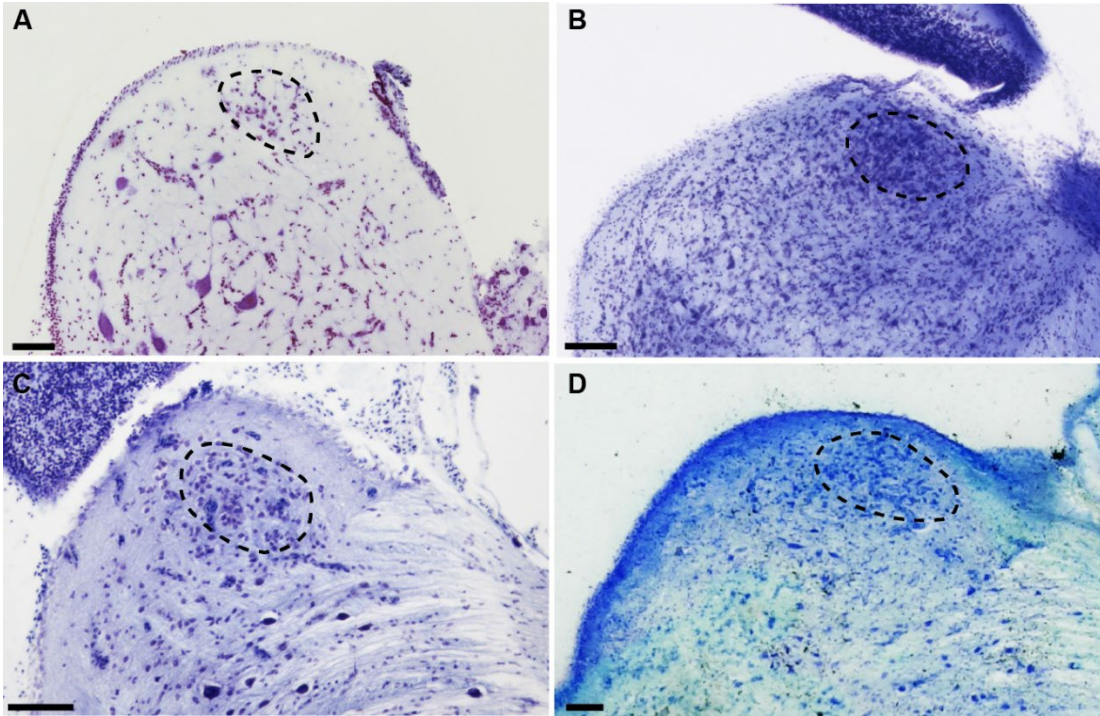


**Figure 3.7 Transverse section of VeO in different lepidosaurs**

A. Tuatara (*Sphenodon punctatus*); courtesy of Jayc Sedlmayr from the collection of Glenn Northcutt

B. Western ratsnake (*Pantherophis obsoletus*)

C. Green anole (*Anolis carolinensis*); courtesy of Catherine McCormick

D. Australian water dragon (*Intellagama lesueurii*); courtesy of the Harvard Museum of Comparative Zoology from the collection of Glenn Northcutt

Left is medial, top is dorsal. Dashed line denotes VeO; scale bar = 100  $\mu\text{m}$  for all panels.

In mammals there is no direct evidence of a sacculo-toral pathway. The saccule, like other vestibular end-organs, projects to hindbrain cochlear nuclei (Burian et al., 1989, 1991; Burian & Gstoettner, 1988; Gstoettner et al., 1991; Newlands et al., 2003; Yingcharoen et al., 2003), but these connections, together with somatosensory input, are thought to aid in suppression of self-generated sounds and to provide information about the orientation of the ear relative to the body (reviewed in Wu et al., 2015). While saccular nerve fibers are sensitive to low-frequency vibrations (reviewed in Curthoys, 2017), in the guinea pig their lowest threshold, measured from afferents innervating striolar hair cells, is approximately 0.02g, equivalent to  $-14 \text{ dB re } 1 \text{ ms}^{-2}$ , at best frequencies (Curthoys et al., 2016), much less sensitive than frogs or the tokay gecko. Recent evidence suggests that spinal cord-inferior colliculus projections, instead of inner ear pathways, facilitate perception of low-frequency vibration in mice (Huey et al., 2024). Fossorial mammals also depend heavily on somatosensation to navigate their environment (e.g. Mason & Narins, 2001; Nevo et al., 1991)

Nevertheless, the presence of VeO in snakes, lizards and the tuatara support the existence of a sacculo-toral circuit mediating vibration in all lepidosaurs and suggests that the ancestral auditory function of the saccule is preserved in this amniote lineage. Early amniotes were atympanic (Clack & Anderson, 2016), and based on evidence in this study, the saccule may have retained the role in sensing vibration in the substrate. Vibrations can also be detected by the cochlea via articulation between the stapes and the quadrate, similar to the mechanism in modern snakes (Han & Carr, 2023), but how cochlear and saccular systems differentiate or

integrate is unclear. Following the appearance of the tympanic membrane, the low-frequency vibration sensitivity from the saccule may have been retained or lost depending on amniote lineage, while the cochlea evolved to sense sound pressure.

### 5. Acknowledgements

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## Chapter 4: Connections and response properties of the snake

### “paratorus”: Extension of Hartline (1971b)

#### 1. Introduction

Hartline (1971b)’s recordings in the torus semicircularis of rattlesnakes represent one of the most important studies of the detection of substrate vibration in snakes. In a series of experiments, Hartline (1971b) characterized two separate pathways that could elicit toral responses: an auditory one involving the VIIIth cranial nerve and inner ear, and a somatic pathway requiring an intact spinal cord. The auditory system was isolated during experiments with destruction of the spinal cord, and the somatic system was isolated by destroying the inner ear. Sensitivity curves based on local field potentials evoked by auditory stimulation were U-shaped, and sharply tuned to the best frequency around 200 Hz. In contrast, the somatic system had a largely flat frequency threshold curve without a distinct best frequency, and with sensitivity that extended beyond the range of the auditory system. Stimuli delivered under the snake’s body, but not the head, generated somatic responses. In a follow-up manuscript (Hartline, 1971a), the somatic system was demonstrated to have a greater dynamic range and a shorter latency than the auditory system. Furthermore, the auditory and somatic responses seemed physically separate. The auditory-response area was small and located ventral to the caudo-medial region of the somatic-responsive area.

The torus semicircularis of snakes differs from that of their lizard relatives (Senn, 1969, 1979; Senn & Northcutt, 1973). The main portion of the caudal torus

semicircularis in snakes consists of a large “paratorus”, and the central nucleus of the torus semicircularis is a small layer or lamina of cells ventral to the overlying “paratorus” and close to the midline. The descriptive schematic in Hartline (1971b) suggests that the somatic area corresponds to the “paratorus” and the auditory area the central nucleus. A homologue to the snake paratorus is found in burrowing lizards, although it is smaller (Senn & Northcutt, 1973). The only known input to the paratorus is the nucleus dorsalis myelencephali located in the spinomedullary junction, however its homologue in other vertebrates has not been identified (Schroeder, 1985).

In this chapter, I repeated the experiments from Hartline (1971b) by recording auditory and somatic responses in the torus semicircularis of the western ratsnakes and used tract tracing to label recordings sites and identify their connections, bridging the earlier physiology and anatomical studies. Single unit activity was recorded to complement the local field potential data reported in Hartline (1971b). Additionally, I review vertebrate literature to determine possible homologues of the snake paratorus and its inputs, and speculate on the evolution of the snake paratorus.

## 2. Material and methods

A total of 17 western ratsnakes [*Pantherophis obsoletus*, snout-vent length  $92.14 \pm 23.57$  cm (n=14), weight  $237.89 \pm 149.07$  g (n=9)] were obtained commercially for this study. Snakes were kept in a 12:12-hour light-dark cycle with 25-30 °C temperature range and offered mice and water ad libitum. Anesthesia and animal

handling protocols were approved by the University of Maryland College Park Animal Care and Use Committee.

## 2.1 Tract tracing and histology

10 snakes were used for tract tracing. Snakes were anesthetized with isoflurane (5%) until fully anesthetized (determined by loss of muscle tone, lack of righting reflex, and lack of reflex to tail pinch). To label connections the torus semicircularis (TS), we injected 10% biotinylated dextran amine (BDA; equal parts of 3k MW and 10k MW; Invitrogen, Carlsbad, CA, USA) in 0.01M PBS or 3% Neurobiotin (NB; Vector Laboratories, Burlingame, CA) in 0.3M NaCl iontophoretically using an alternating positive current of 2  $\mu$ A for 10-30 minutes. Tracer injections into the torus TS were made *in vivo*. One snake received destructive surgery of the inner ear during physiology (see below) and was perfused transcardially with oxygenated ice-cold Ringer's solution [96.5mM NaCl, 31.5mM NaHCO<sub>3</sub>, 4mM CaCl<sub>2</sub>, 2.6mM KCl, 2mM MgCl<sub>2</sub>, and 20mM D-glucose, from Kohl et al. (2014)], then rapidly decapitated. The isolated head was immersed in oxygenated Ringer's solution in an 8-10 °C refrigerator for 24-48 hours, with oxygenated Ringer's solution changed every 12-24 hours, to enable tracer transport. The other snakes (n=9) were allowed to recover from anesthesia, and were euthanized, perfused and decapitated 48 hours later. The head was immersion-fixed in 4% paraformaldehyde (PFA) in 0.01M phosphate buffered saline (PBS) for 24 h. The brain was extracted and cryoprotected for 24 h in 30% sucrose in 0.01M PBS. Coronal brain sections were cut on a freezing microtome at 50  $\mu$ m. To visualize

tracers, free floating sections were washed and incubated in avidin-biotin complex (ABC; Vector Laboratories, Burlingame, CA) and then stained with SG chromogen (Vector Laboratories). Sections were mounted on gelatin coated slides, air dried and counterstained with Neutral Red. Images were photographed with Neurolucida (MBF Bioscience, Williston, VT, USA) in conjunction with a light microscope (Olympus BX60).

One additional snake was processed for Nissl staining to determine the cytoarchitecture of the torus semicircularis. After fixation, cryoprotection, sectioning and mounting the brain as described above, slides were then stained with cresyl violet, coverslipped, and imaged.

## 2.2 Electrophysiology

In vivo recordings from 9 snakes were used to investigate the response properties of TS. Snakes were anesthetized in an induction chamber with 5% isoflurane anesthesia, then intubated. A constant flow of carbogen mixed with 1-3% isoflurane at 2-4 ml/min was maintained via a tube into the trachea, and respiration continued under these conditions. A dorsal craniotomy exposed the posterior midbrain. We glued a stainless-steel head post to the prefrontal bone to hold the head in a constant position on a custom-made platform.

Pure tone dorsal-ventral vibration stimuli (20-1000 Hz) were produced with a vibration exciter (Type 4810; Brüel & Kjær, Nærum, Denmark) connected to the platform. Tonal stimuli had a duration of 250 ms, including 30 ms cosine onset and offset ramps, with an interstimulus interval of 200 ms (8 animals), or duration of 600

ms, including 100 ms cosine onset and offset ramps, with an interstimulus interval of 400 ms (1 animal). The vibration exciter was calibrated using a piezoelectric accelerometer (Type 4381; Brüel & Kjær, Nærum, Denmark) placed on the platform next to the snake. The accelerometer was connected to a conditioning amplifier (Type 1704; Brüel & Kjær). The accelerometer itself was calibrated using a calibration exciter (Type 4294; Brüel & Kjær) at  $10 \text{ ms}^{-2}$ , 159.15 Hz with fixed attenuation to levels around -13 dB re  $1 \text{ ms}^{-2}$ . Pure-tone vibration stimuli (10 Hz- 1000 Hz) was generated using custom-written MATLAB software (*tytology*). Signals were fed through a processing device (RX8; Tucker-Davis Technologies, Alachua, FL) and a programmable attenuator (PA5; Tucker-Davis Technologies). Stimulus levels ranged from -53 dB re  $1 \text{ ms}^{-2}$  to 2 dB re  $1 \text{ ms}^{-2}$ .

Recordings were obtained with tungsten microelectrodes (F. Haer, Bowdoin, ME) with impedances around 20 M $\Omega$ . The location of TS was estimated based on histological sections. Electrodes over the caudal midbrain and advanced using a micro-drive system ( $\mu$ D-800; Walsh) while continuously testing for responses to vibration using different pure tone stimuli. Recorded extracellular signals were amplified ( $\mu$ A-801D; Walsh Electronics) and bandpass-filtered (300-12000Hz), passed through a Hum Bug (Quest Scientific, North Vancouver, BC) to eliminate electrical interference, and converted from analog to digital at 48.8 kHz (RX8). Recordings were visualized online with *tytology* (Go Ashida, Sharad Shanbhag). We measured rate-intensity functions with co-varying frequency and level, with 5 or 10 repetitions per stimulus. Tones of different frequencies and levels were presented in random order. We limited the number of repetitions to 5-10 because of concerns

about minimizing electrode drift. Each locus also included multiple ( $> 40$  repetitions) duration-matched recordings of spontaneous activity.

To characterize somatic units, both otic capsules of a snake ( $n=1$ ) were opened dorsally, and the membranous labyrinths were carefully removed. This surgery was terminal. Vibration stimuli were delivered to the midsection of the snake's body. In the same animal, we also tested whether responses could be found in the torus when vibration stimuli were delivered to the snake's head, and found none, suggesting head vibration responses are auditory. To characterize auditory units, vibration stimuli were delivered under the head ( $n=8$ ). BDA or NB was injected in the recording site to label the recording site in the torus and trace its connections. Three cases yielded sufficient retrograde transport to the brainstem or spinal cord.

Background noise on the shaker platform caused by environmental vibrations and electrical noise from the amplifier was measured for all stimulus frequencies. To measure noise, we made 80 recordings with a time window of 250 ms or 600 ms corresponding to the duration of one stimulus. Each recording was windowed by a Tukey window ( $\alpha=0.1$ ) and filtered by a third-order Butterworth filter with cutoff frequencies corresponding to one octave band around center frequency. The noise level was then calculated as r.m.s. noise level for the filtered 250 ms and 600 ms recording.

Signals were further processed offline using a combination of custom-written MATLAB software and UltraMegaSort2000 (D. N. Hill et al., 2011). Putative spikes were detected by threshold of the voltage trace of all recordings done at the same location at 2.3 times the standard deviation with a 0.6 ms shadow period. Events were

then overclustered using k-means and merged based on interface energy between clusters (D. N. Hill et al., 2011). Putative single units were manually inspected based on the general spike waveform, estimated number of subthreshold spikes, inter-spike interval refractory period violations and autocorrelation function. 33 out of 41 recordings contained well-isolated single units, and the remaining were counted as multi-unit recordings.

We used custom written MATLAB software to analyze spike data. Threshold levels for each frequency were determined as 2 spikes/s above the estimated spontaneous rate and increase in firing rate in the next stimulus level (+10 dB). Best frequency (BF) was determined as the frequency with the lowest threshold. We measured phase-locking using a period histogram, where the instantaneous firing rate was plotted as a function of stimulus phase in cycle. Degree of phase-locking was quantified using vector strength (Goldberg & Brown, 1969). Interspike interval histograms and peristimulus time histograms for all spikes during the stimulus were calculated and plotted.

### 3. Results

The description of western ratsnake torus semicircularis will follow terminology introduced in (Senn, 1969), where the posterior midbrain of snake comprises the “torus semicircularis” and “paratorus”. The “torus semicircularis” in Senn (1969) is also identified in lizards and corresponds to the central nucleus of the torus semicircularis in other lizard midbrain literature (Browner & Robinson, 1977; Foster & Hall, 1978; Yan et al., 2010). To avoid confusion, the torus semicircularis of

the western snakes will be separated into a central nucleus and a paratorus, the former corresponding to the “torus semicircularis” in Senn (1969), Senn (1979) and Senn & Northcutt (1973). The central nucleus is continuous with the periventricular gray of the optic tectum, whereas the paratorus is continuous with the central gray of the optic tectum (Senn, 1969, 1970, 1979; Senn & Northcutt, 1973). The term “central gray” used here and in Senn’s works refers to the gray matter layers intermediate between the superficial and periventricular layers, instead of the gray matter directly adjacent to the cerebral aqueduct sometimes known as the “central gray”.

The paratorus embodies the entire caudal pole of the torus semicircularis and extends medially to join the homologous nucleus on the opposite side. In more rostral transverse sections (Figure 4.1A), neuropil continuous with the periventricular grey appears medial and ventral to the paratorus, containing the ventrally located central nucleus of the torus and the dorsally located caudal pole of the mesencephalic trigeminal nucleus (Me5). The periventricular layer thus separates the adjoining paratorus from the central nucleus. More rostrally the torus bifurcates to form two wings below the tectal ventricles (Figure 4.1E). At this level, the central nucleus in non-ophidian reptiles is often ovoid or spherical with loosely arranged cells (Figure 4.1F), sometimes forming a distinct protrusion into the tectal ventricles (Browner et al., 1981; Knudsen, 1983; Pritz, 1974; Yan et al., 2010), whereas in the western ratsnake the central nucleus remains as a neuron-dense layer structure with indistinct boundaries. The paratorus gradually decreases in size in the rostral midbrain to become indistinguishable in the central gray.

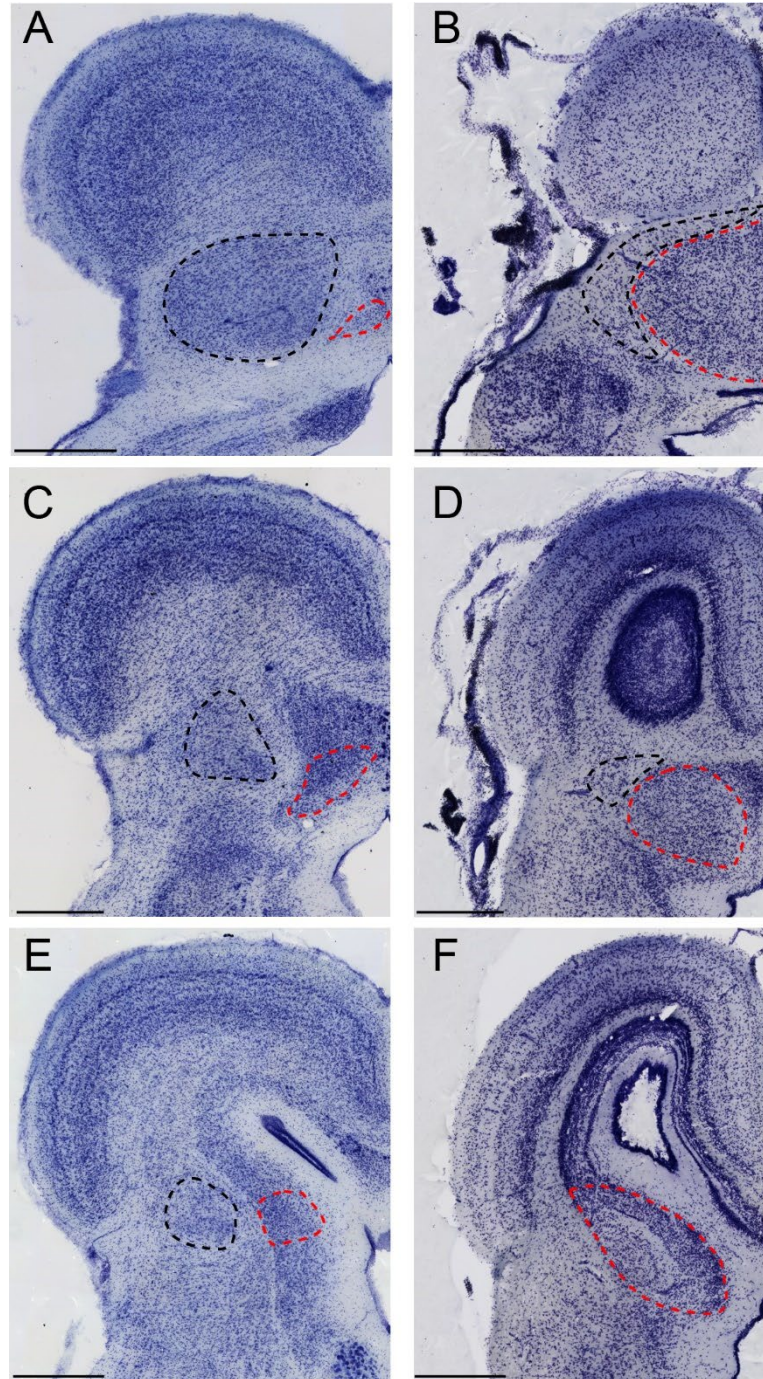


**Figure 4.1 The snake paratorus and its lizard counterpart**

Transverse sections of the midbrain of the western ratsnake (A, C, E) and the tokay gecko (B, D, F). Black dashed lines outline the boundaries of the paratorus in the western ratsnake and the superficial toral nucleus in the tokay gecko. Red dashed lines outline the boundaries of the central nucleus of the torus semicircularis. The boundaries of the snake central nucleus in E are indistinct. Top to bottom, caudal to rostral. Scale bar = 500  $\mu\text{m}$ .

### 3.1 Connections of the torus semicircularis

Small injections contained within the paratorus reveal reciprocal connections with the contralateral homologue via the commissural tract dorsal to Me5 (Figure 4.2A, D). Retrogradely labelled neurons were also seen consistently in the ipsilateral central nucleus (Figure 4.2B).

Injections in the paratorus also labelled structures in the brainstem auditory pathway. Lateral lemniscus fibers exited the paratorus from the ventrolateral aspect (Figure 4.2B), travelling close to the lateral surface of the brain and labelled neurons and terminals in the dorsal nucleus of the lateral lemniscus (LLD; Figure 4.3A) and the superior olivary nucleus (SO; Figure 4.3B), both mainly on the ipsilateral side. Fibers from the paratorus also travelled to the contralateral side via the commissural tract dorsal to the Me5, caudal to the formation of the tectal ventricles in transverse sections, and passed through the contralateral paratorus to form sparse connections with the contralateral LLD and SO. In most cases, neurons in the contralateral acoustic tubercle were labelled, including neurons in nucleus angularis (NA; Figure 4.3C, D), nucleus vestibularis ovalis (VeO; Figure 4.3E) and an undescribed area medioventral and ventral to VeO (Figure 4.3F). In one case, an ipsilateral NA neuron was labelled.

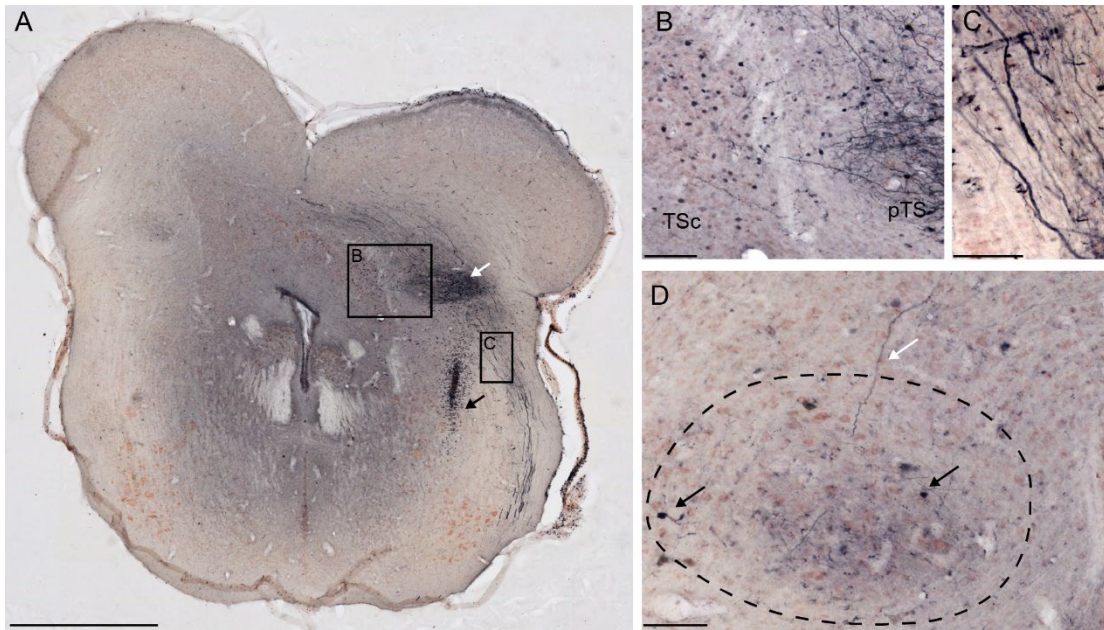


**Figure 4.2 Midbrain connections of the paratorus**

A. Tracer injection site. The right side is slightly distorted due to pressure from the recording electrode. Electrode tract (black arrow) reached below the injection site (white arrow). The boxes indicate areas in B and C.

B. Labelled neurons in the central nucleus (TSc) following injection in the paratorus (pTS). Axons from TSc neurons could be seen extending into paratorus.

C. Labelled axons connecting the paratorus with brainstem and spinal cord neurons. Thick axons originate from neurons in the spinal cord and nucleus dorsalis myelencephali, and thin axons originate from auditory nuclei.

D. Labelled neurons (black arrows) and fibers (white arrow) in the contralateral paratorus (dashed line) in a more caudal transverse section.

Scale bar = 1 mm in A; 100  $\mu$ m in B-D.

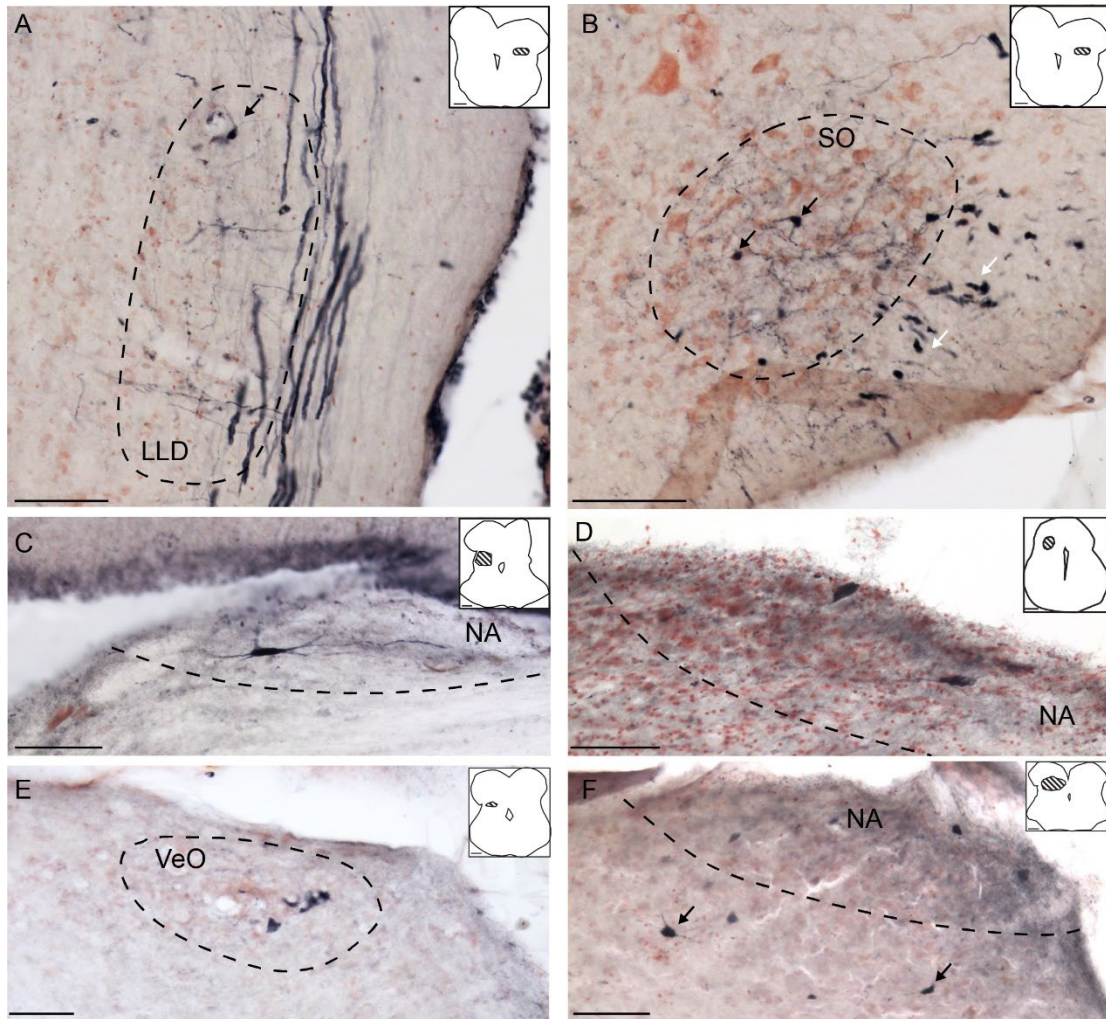


**Figure 4.3 Auditory connections of the paratorus**

A. Labelled fibers, terminals and neuron (arrow) in the dorsal nucleus of the lateral lemniscus (LLD; dashed line). Thin fibers can be seen exiting LLD horizontally.

B. Labelled fibers, terminals and neurons (black arrows) in the superior olivary nucleus (SO). Cross-section of thick spinal lemniscal fibers (white arrows) can be seen superficial (lateral) to SO.

C. Retrogradely labelled neuron in rostral nucleus angularis (NA; dashed line).

D. Retrogradely labelled neurons in more caudal NA (dashed line).

E. Retrogradely labelled neurons in nucleus vestibularis ovalis (VeO).

F. Retrogradely labelled neurons in NA (dashed line) following a large injection in the paratorus and central nucleus. Additional labelled neurons were seen ventromedial and ventral to NA (arrows). The same neurons were also seen in case with injection only in the paratorus.

For all figures, left is medial, top is dorsal. Insets show injection sites (diagonal lines). Scale bar = 100  $\mu\text{m}$  for figures and 500  $\mu\text{m}$  for insets.

Injections in the paratorus consistently labelled structures in the lower hindbrain (or the spinomedullary junction), known as the nucleus dorsalis myelencephali (DM; Schroeder, 1985) and spinal cord. Spinal lemniscus fibers exited the paratorus from the ventrolateral aspect, intermixed with lateral lemniscus fibers, but were noticeably thicker (Figure 4.2C). Injections labelled neurons in the parvocellular area of DM (Schroeder, 1985). Label was more prominent on the contralateral DM compared to ipsilateral (Figure 4.4A, B), and axons from DM crossed the gray matter of the spinal cord under the central canal to ascend in the spinal lemniscus (Figure 4.4A). Labelled lateral funiculus fibers were seen beyond DM in the spinal cord, and their origins are unknown. In some animals, a few labelled neurons were found in the gray matter of the spinal cord corresponding to medial lamina VI or lamina V (Figure 4.4C, D). No label was found in the dorsal column nuclei.

In one case with large injections of tracer into the caudal midbrain that included the paratorus and parts of the central nucleus, the same structures were labelled as described above. There were more neurons labelled in the acoustic tubercle (Figure 4.3F), but we are uncertain if this was due to spread of tracer in the central nucleus or simply from a large injection. We were unable to make dye injections in only the central nucleus, given the small area of the central nucleus in the western ratsnake.

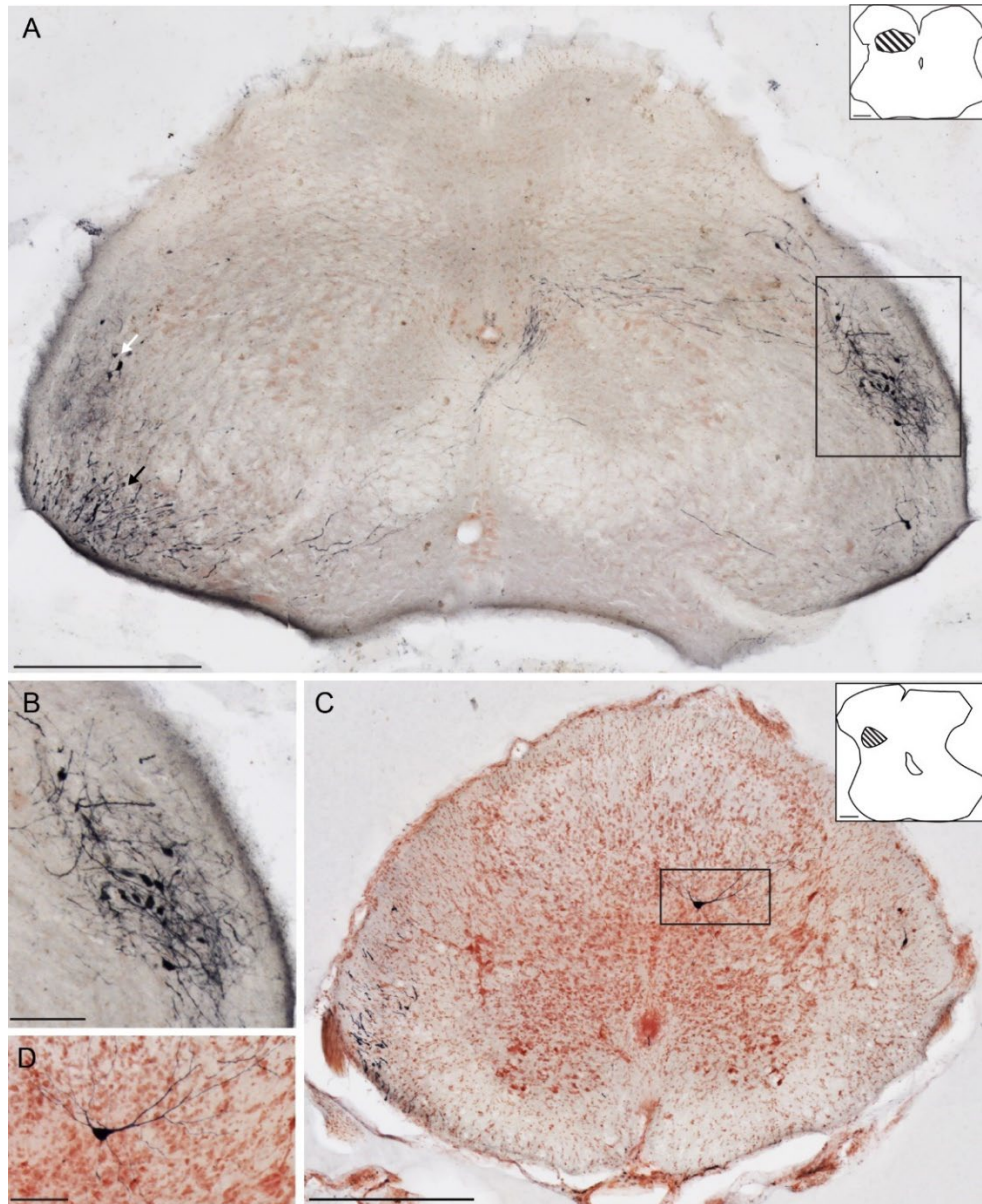


**Figure 4.4 Somatic connections to the paratorus**

A. Retrograde label in nucleus dorsalis myelencephali (DM) at the level of the spinomedullary junction. Label neurons were mainly in the contralateral DM (box) compared to the ipsilateral side (white arrow). Fibers from the spinal cord that project to the paratorus were located mainly in the ipsilateral lateral funiculus of the spinal cord (black arrow, also in C). The box indicates area in B.

B. High power microphotograph of the contralateral DM.

C. Retrogradely labelled cell in the gray matter of the spinal cord. Black arrow indicates fibers in the spinotor tract in the lateral funiculus. The box indicates area in D.

D. High power microphotograph of paratorus projection neuron in the spinal gray.

Scale bar = 500 µm for A, C; 100 µm for B, D; 500 µm for insets.

### 3.2 Physiological responses in the paratorus

We characterized the responses of paratorus neurons to vibration using extracellular electrophysiological recordings. Paratorus units were sensitive to low frequency vibration detected by the auditory system or somatic system.

A total of 25 single units and 8 multi-units from 8 animals responded to auditory stimuli (Figure 4.5A). Best frequencies (BF) ranged from 40 Hz to 300 Hz, with BF for most units around 100 Hz. Mean threshold measured at BF was  $-27.60 \pm 11.45$  dB re  $1 \text{ ms}^{-2}$ . The lowest single unit threshold was -48 dB for one unit with BF of 100 Hz. The highest single unit threshold was -13 dB for one unit with BF of 80 Hz. Vector strengths were measured at BF at 10 dB above threshold (n=22) if allowed, or 5 dB above threshold (n=2) or at threshold (n=1) for units with high thresholds, and varied from 0.04 to 0.98, with an average of  $0.29 \pm 0.26$  (Figure 4.5C). In our analysis of frequency tuning, we included data from an additional 8 multi-unit recordings from 3 animals where we were unable to isolate single units based on spike sorting criteria (Figure 4.5A). BFs ranged from 80 to 100 Hz, and mean vibration thresholds measured at BF were  $-36.75 \pm 9.16$  dB  $1 \text{ ms}^{-2}$ . The lowest multi-unit thresholds were -53 dB for four units with best frequencies of 80 Hz and 100 Hz. The highest multiunit threshold was -18 dB for one unit with a best frequency of 100 Hz. Onset type units generally had a higher threshold, which may contribute to the higher mean thresholds in single units compared to multi-units (see below). Ambient noise levels in one-octave bands around all tested frequencies were around -90 dB re  $1 \text{ ms}^{-2}$ , or at least 47 dB below threshold.

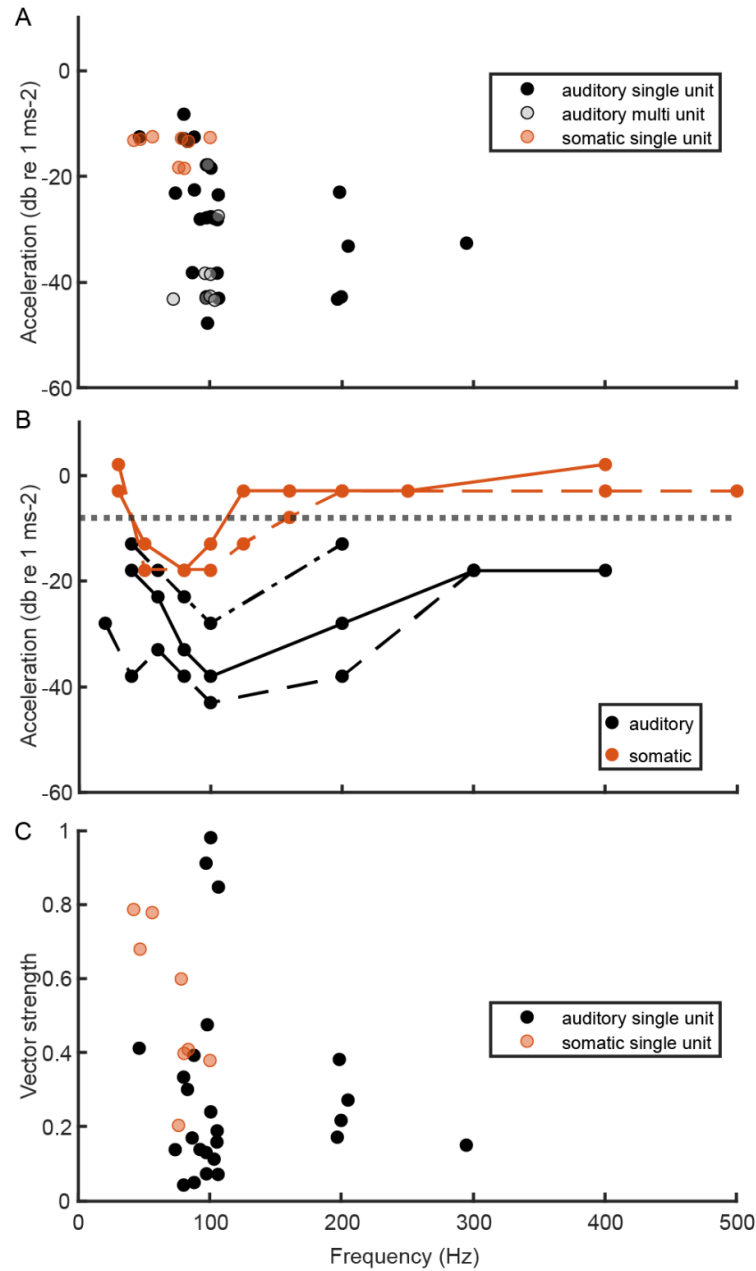


**Figure 4.5 Physiological properties of paratorus neurons**

A. Vibration thresholds measured at BF in paratorus, with data from 25 auditory single units, 8 auditory multiunit, and 8 somatic single units.

B. Tuning curve examples for 3 auditory single units and 2 somatic single units. The grey dotted line represents the maximum stimulus level used for auditory units (-8 dB).

C. Vector strength (VS) values for single units measured at BF at 10 dB above threshold (n=22) or 5 dB above threshold (n=2) or at threshold (n=1).

The response characteristics of paratorus auditory units were varied. There was an abundance of onset units, characterized by a single prominent peak in their PSTH (Figure 4.6). Most onset units had poor phase-locking (Figure 4.6A-C), although a few were entrained to the stimulus (Figure 4.6D-F). In general, spontaneous firing rate was low ( $<2$  spikes/s), however one unit had a relatively high spontaneous firing rate (3.3 spikes/s) and the PSTH showed a delayed onset response ( $\sim 40$ ms) relative to stimulus onset, and decreased discharge rates below the spontaneous rate during other parts of the stimulus (Fig. 4.6G-I). Additionally, there were primary-like units, characterized by vigorous discharge at stimulus onset that gradually adapted to a steady lower discharge (Fig 4.7). We also found a phase-locked, tonically firing unit with a steady discharge rate (Fig 4.8), and a unit that had a sustained response with a delayed onset ( $\sim 100$ ms; Fig 4.9). Additionally, we encountered units with irregular firing patterns, showing a clear onset response at low stimulus levels, but at high levels the onset response is followed by a cessation of activity and then lowered (compared to onset) but steady discharge rates (Fig 4.10). The duration of the pause was dependent on stimulus level, i.e. at higher stimulus level the pause was smaller, and at lower levels the neuron fired like an onset unit.

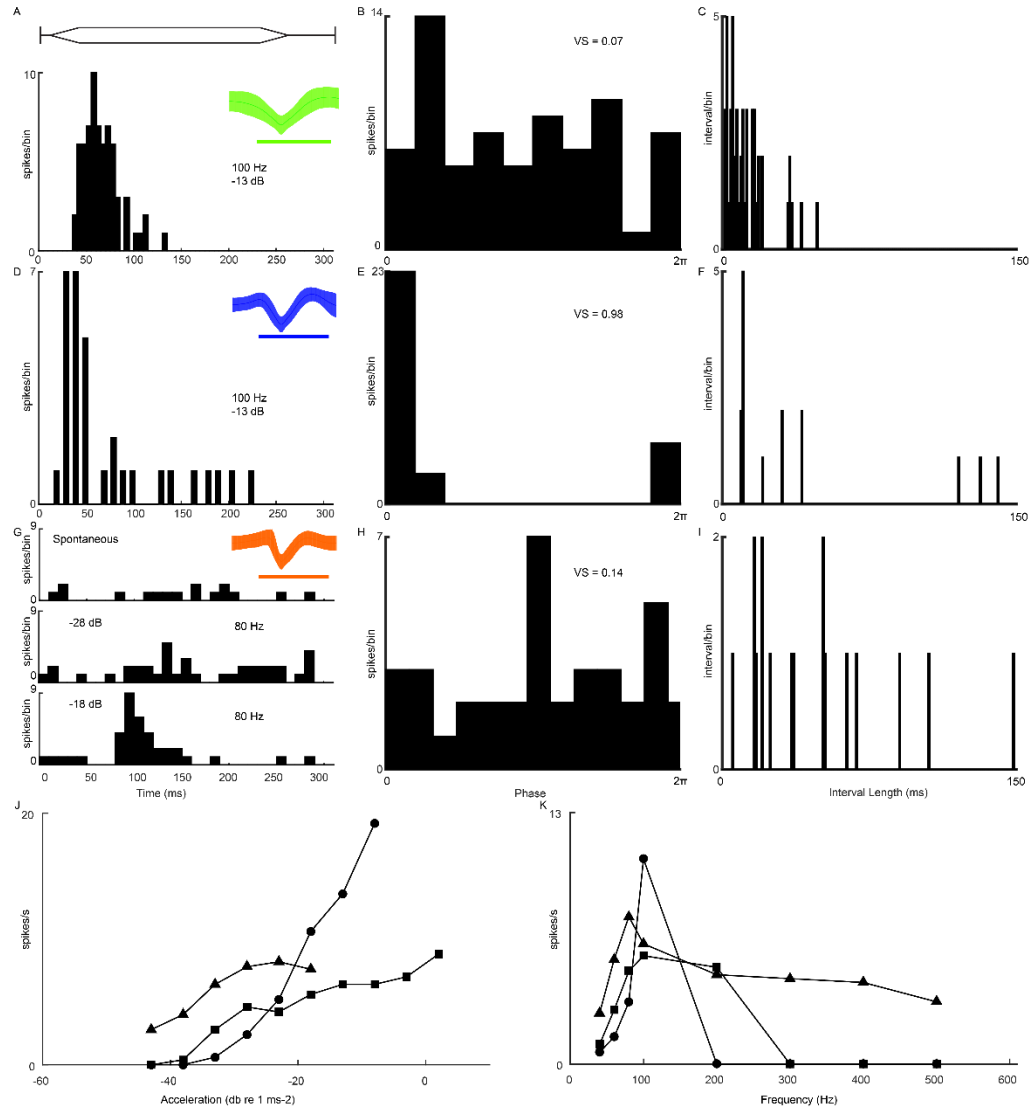


**Figure 4.6 Example of auditory units with onset responses**

A-C Onset unit with poor phase locking.

A. PSTH at BF = 100 Hz, -13 dB re  $1 \text{ ms}^{-2}$  = 10 dB above threshold. Top: time parameters of the vibrational stimuli. Bin width = 0.05 ms. Inset: spike waveform, scale bar = 1 ms.

B. Period histogram with the same stimulus. Vector strength (VS) = 0.07.

C. Interspike interval histogram with the same stimulus.

D-F Onset unit with good phase locking.

D. PSTH at BF = 100 Hz, -13 dB re  $1 \text{ ms}^{-2}$  (15 dB above threshold). Inset: spike waveform, scale bar = 1 ms. Bin width = 0.05 ms. The “gaps” in the PSTH indicate phase-locking, since the bin width equals the half cycle of the stimulus.

E. Period histogram with the same stimulus. Vector strength (VS) = 0.98.

F. Interspike interval histogram with the same stimulus.

G-I Unit with delayed onset.

- G. PSTH with no stimulus, at BF = 80 Hz, -28 dB (at threshold) and at BF, -18 dB (10 dB above threshold). Bin width = 0.1 ms. Inset: spike waveform, scale bar = 1 ms.
- H. Period histogram at BF, -18 dB.
- I. Interspike interval histogram with the same stimulus.
- J. Rate-level functions of the three units, measured at BF. Circle, unit in A-C; square, unit in D-F; triangle, unit in G-I.
- K. Iso-intensity (-18 dB) frequency-rate curves for the three units. Circle, unit in A-C; square, unit in D-F; triangle, unit in G-I.

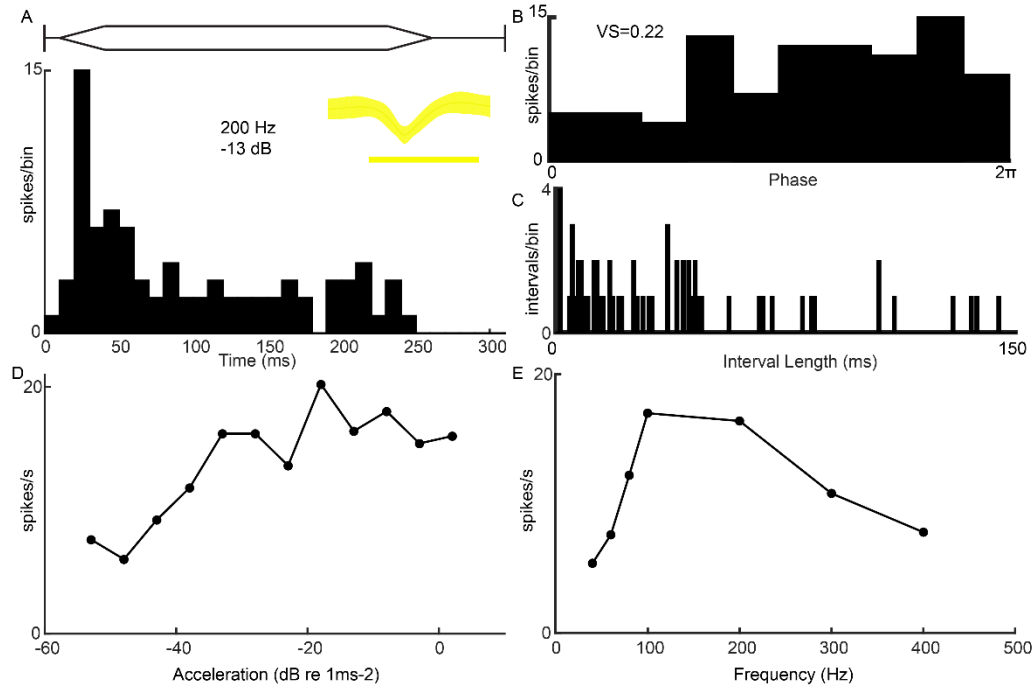


**Figure 4.7 Example of an auditory unit with a primary-like response**

- A. PSTH at BF = 200 Hz, -13 dB re 1 ms<sup>-2</sup> = 30 dB above threshold. Top: time parameters of the vibrational stimuli. Inset: spike waveform, scale bar = 1 ms.
- B. Period histogram with the same stimulus.
- C. Interspike interval histogram with the same stimulus.
- D. Rate-level function measured at BF.
- E. Iso-intensity (-13 dB) frequency-rate curve.

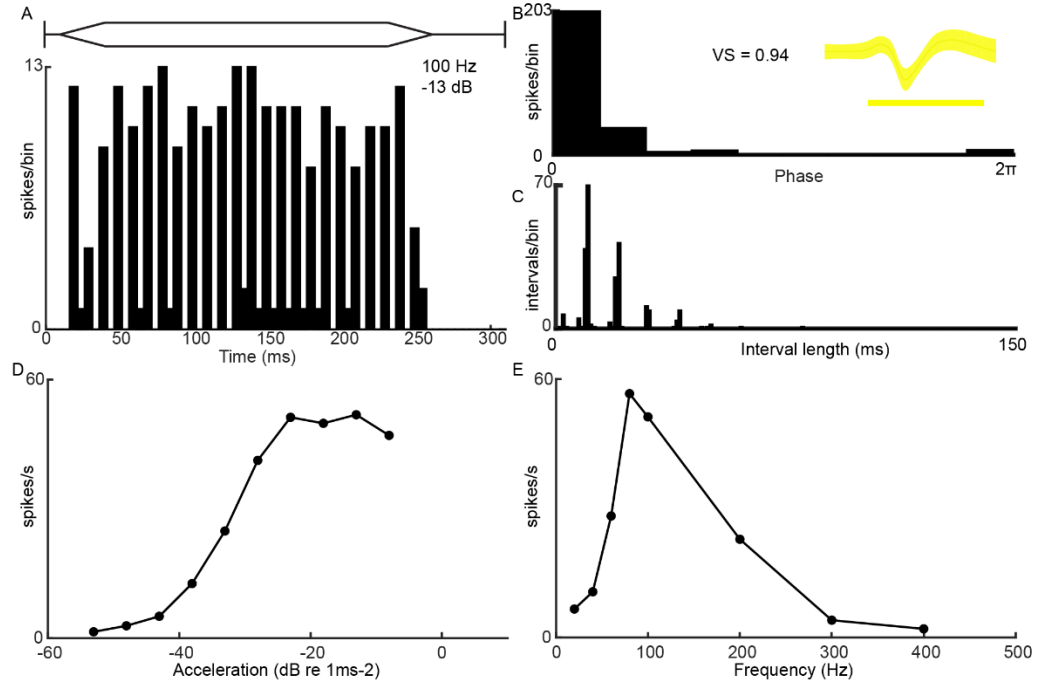


**Figure 4.8 Example of an auditory unit with a tonic response**

- A. PSTH at BF = 100 Hz, -13 dB re  $1 \text{ ms}^{-2} = 30 \text{ dB}$  above threshold. Top: time parameters of the vibrational stimuli.
- B. Period histogram with the same stimulus. Inset: spike waveform, scale bar = 1 ms.
- C. Interspike interval histogram with the same stimulus. VS = 0.94.
- D. Rate-level function measured at BF.
- E. Iso-intensity (-13 dB) frequency-rate curve.

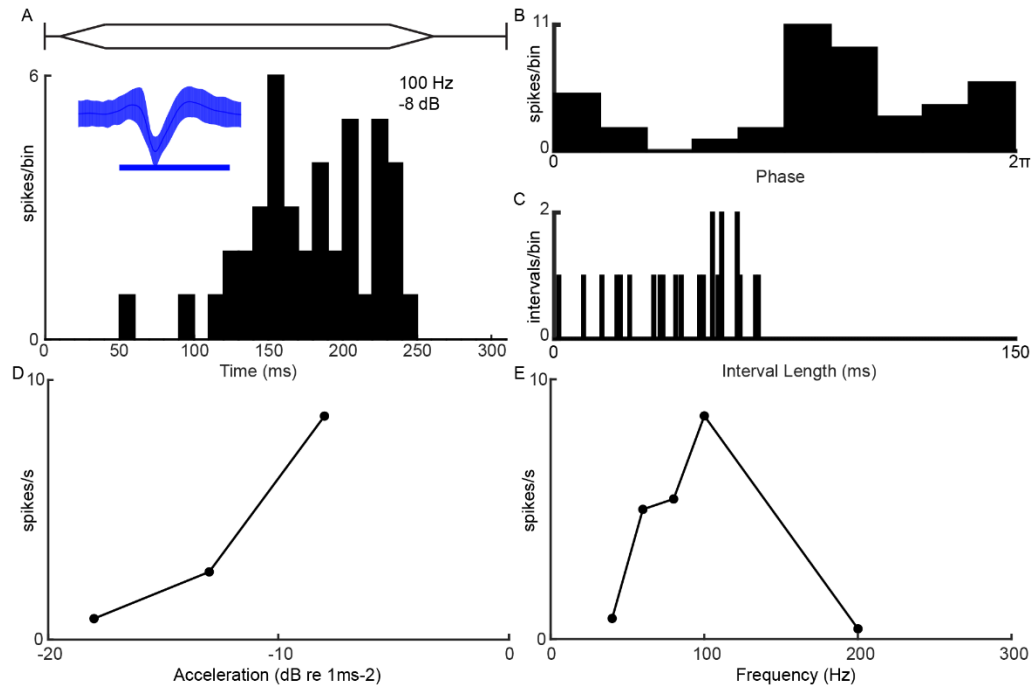


**Figure 4.9 Example of an auditory unit with a delayed sustained response**

- A. PSTH at BF = 100 Hz, -8 dB re  $1 \text{ ms}^{-2} = 5 \text{ dB}$  above threshold. Top: time parameters of the vibrational stimuli. Inset: spike waveform, scale bar = 1 ms.
- B. Period histogram with the same stimulus.
- C. Interspike interval histogram with the same stimulus.
- D. Rate-level function measured at BF.
- E. Iso-intensity (-8 dB) frequency-rate curve.

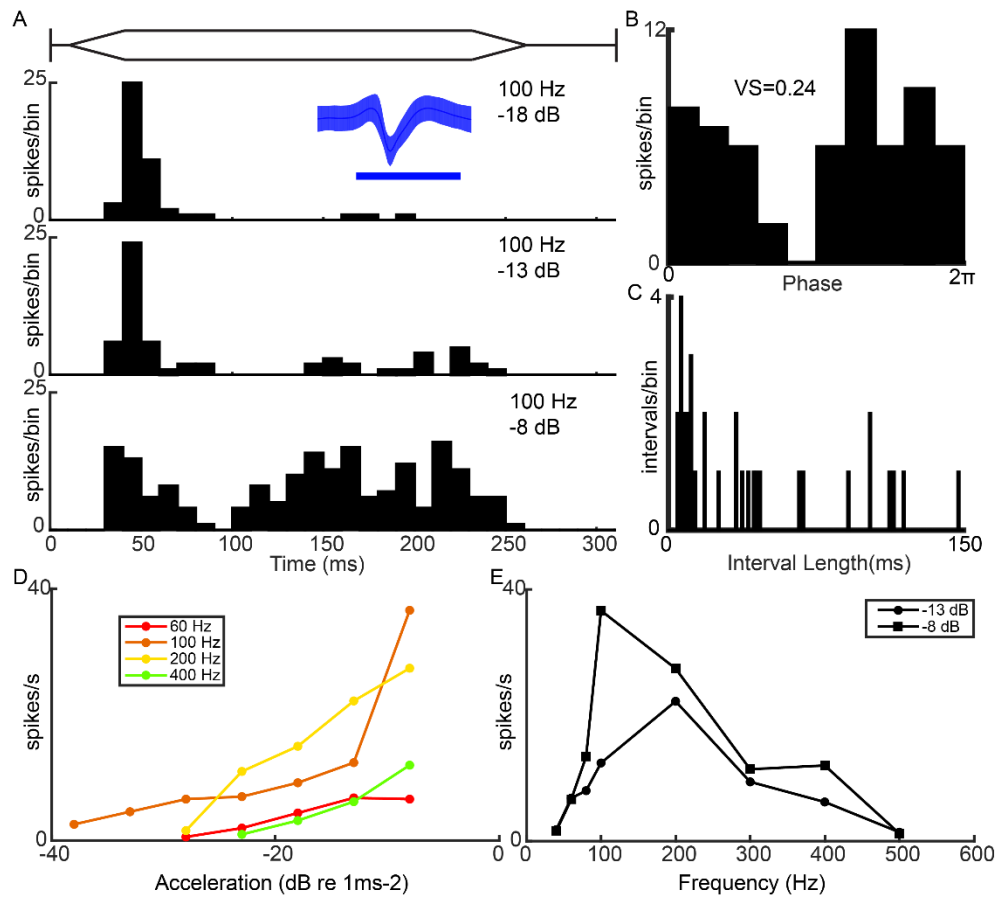


**Figure 4.10 Example of an auditory unit with irregular firing**

A. PSTH at BF = 100 Hz, -18 dB (20 dB above threshold), -13 dB (25 dB above threshold) and -8 dB (-8 dB above threshold). Top: time parameters of the vibrational stimuli. Inset: spike waveform, scale bar = 1 ms.

B. Period histogram at BF, -13 dB.

C. Interspike interval histogram with the same stimulus.

D. Rate-level function measured at 60 Hz (red), 100 Hz (orange), 200 Hz (yellow), and 400 Hz (green)

E. Iso-intensity frequency-rate curve at -13 dB (circle) and -8 dB (square).

A total of 8 single units from 1 animal responded to somatic stimuli. Best frequencies ranged from 50 Hz to 100 Hz (Fig 4.5A). Mean threshold measured at BF was  $-14.25 \pm 2.31$  dB. The lowest threshold was -18 dB for two units with BF at 80 Hz. Somatic units responded to higher frequencies (300 Hz-600 Hz) at around -2 dB. Vector strength measured at 10 dB above threshold at BF varied from 0.20 to 0.79, with an average of  $0.53 \pm 0.21$  (Figure 4.5C).

The somatic units shared similar firing patterns, although our current sample size is small. Response patterns were similar to the irregular-firing auditory unit but differed from the auditory unit that at frequencies above BF, where the neurons maintained onset responses, even at high stimulus levels (Fig 4.11).

We did not test whether units responded to both auditory and somatic stimuli. While head vibrations can be considered pure auditory stimuli, body vibrations excite the somatic and auditory systems, due to conduction of vibration from the body to the inner ear (Hartline, 1971b). Testing the response to both auditory and somatic stimuli would require two vibration exciters and surgical destruction of the inner ear while maintaining electrode position.

Based on electrode tracts and dye injections at recording sites, we did not record any responses in the central nucleus. The locations of somatic and auditory responses were all in the paratorus and seemed to overlap in distribution.

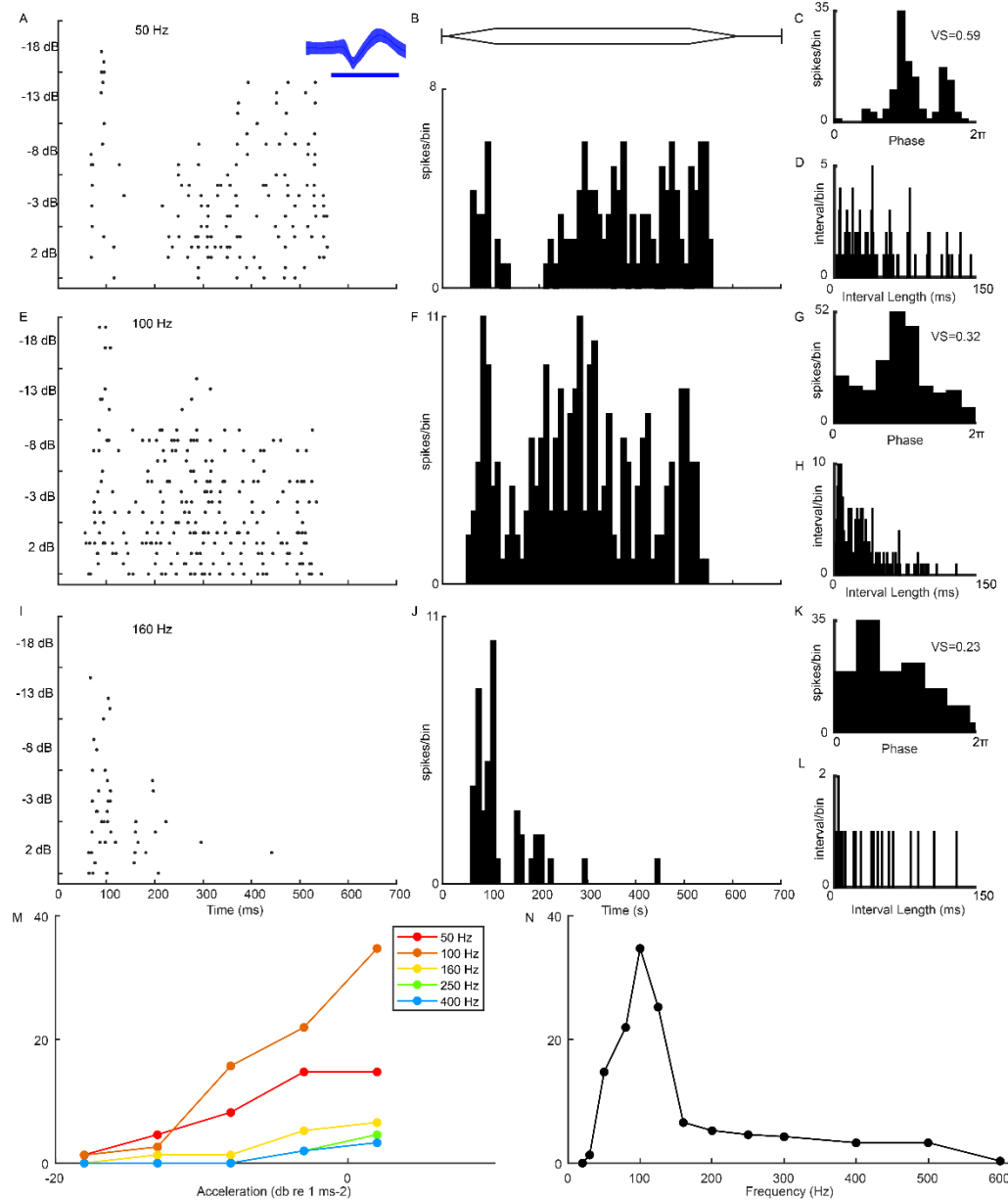


**Figure 4.11 Example of a somatic unit**

Dot raster plots include responses from -18 dB to -2 dB re  $1 \text{ ms}^{-2}$ , 5 repetitions each stimulus level. PSTH, period histograms and interspike interval histograms combine data from -43 dB to -18 dB, 5 repetitions each stimulus level, 25 repetitions total.

A-C Response characteristics at 50 Hz.

A. Dot raster plot. Inset: spike waveform, scale bar = 1 ms.

B. PSTH. Top: time parameters of the vibrational stimuli.

C. Period histogram.

D. Interspike interval histogram.

E-H Response characteristics at BF = 100 Hz.

E. Dot raster plot.

- F. PSTH.
- G. Period histogram.
- H. Interspike interval histogram
- I-L Response characteristics at 50 Hz.
- I. Dot raster plot.
- J. PSTH.
- K. Period histogram.
- L. Interspike interval histogram
- M. Rate-level functions of the three units, measured at 50 Hz (red), 100 Hz (orange), 160 Hz (yellow), 250 Hz (green), and 400 Hz (blue).
- N. Iso-intensity (2 dB) frequency-rate curve.

#### 4. Discussion

The torus semicircularis of the western ratsnake is separated into a small, laminar central nucleus and a large, laterally situated paratorus, confirming observations of Senn (1969). The paratorus receives input from cochlear nuclei, the spinal cord and the nucleus dorsalis myelencephali (DM; Schroeder, 1985), and is reciprocally connected with its contralateral homologue. The central nucleus projects to the paratorus. Its connection and location are similar to the midbrain somatosensory regions in the other vertebrate species. Neurons in the paratorus are sensitive to low frequency vibration detected by the auditory and somatic systems and show heterogenous response types. Recent studies suggest the midbrain somatosensory area in mammals is related to vibration detection (Huey et al., 2024); a hypertrophied midbrain somatosensory area in snakes indicates enhanced computational performance for vibration sensation. The evolution of a large paratorus is likely related to the loss of the tympanic middle ear and the potential shift to a burrowing lifestyle during snake evolution.

##### 4.1 Midbrain somatosensory areas in other vertebrates and their connections

The paratorus is characterized by significant projections from spinal pathways and by its location in the central gray of the torus semicircularis. While the term “paratorus” has only been used in snakes and in burrowing lizards in the context of their relationship to snakes (Senn, 1969, 1979; Senn & Northcutt, 1973), comparable structures have been identified in other vertebrates.

In lizards, the intercollicular nucleus is located in the central gray matter lateral to the central nucleus and ventral to the optic tectum (hence the name “intercollicular”), and terminal degeneration in the intercollicular nucleus was observed following lesions in the spinal cord (tegu: Ebbesson, 1967; iguana: Foster & Hall, 1978). The intercollicular nucleus corresponds to the lateral region of the superficial nucleus in the tokay gecko (Kennedy & Browner, 1981; Figure 4.1B, D, F). The intercollicular nucleus was homogenized with the “caudolateral portion of the central stratum of the torus semicircularis” in the lizard *Gallotia galloti* (Díaz et al., 2000), affirming its location in the central grey and comparability with the snake paratorus. In lizards and also turtles, cells that would later form the central grey matter migrated from an early periventricular layer during embryonic development (Senn, 1970). In the green iguana, the intercollicular nucleus was indicated to make connections with the central nucleus but did not receive input from brainstem cochlear nuclei (Foster & Hall, 1978). The central nucleus receives minor input from the dorsal column nuclei (Ebbesson, 1967). Note that both Ebbesson (1969) and ten Donkelaar & Nieuwenhuys (1979) described an intercollicular nucleus separate from the central nucleus in the *Boa constrictor* and *Python reticulatus*, but failed to recognize its continuation with the caudal pole of the torus semicircularis and incorrectly labelled the caudal paratorus as the central nucleus.

In turtles, the intercollicular nucleus has a similar location and spinal connections to lizards (Ebbesson, 1969). The intercollicular nucleus in turtles was also referred to as the peritoral nucleus (Kunzle & Woodson, 1982). The intercollicular nucleus receives input from, and possibly also projects to, the

ipsilateral central nucleus (Belekhova et al., 1985), consistent with our findings in the snake paratorus. The central nucleus also receives direct spinal or dorsal column nuclei afferents (Belekhova et al., 1985; Kunzle & Woodson, 1982). Neurogenesis of the central nucleus compared to the intercollicular nucleus is delayed in the soft-shell turtle (Zeng et al., 2007).

In the caiman, projections from the spinal cord ascend via the spinal lemniscus to terminate in the intercollicular nucleus and also in the lateral aspect of the central nucleus (Ebbesson & Goodman, 1981; Pritz, 2002; Pritz & Stritzel, 1989). Midbrain projecting neurons were restricted to the dorsal aspect of the dorsal column nuclei (Pritz, 2002). The midbrain somatosensory recipient area in the caiman does not receive input from cochlear nuclei (Pritz, 2002).

Among birds, an intercollicular nucleus of the pigeon was initially described to envelope the central nucleus on all sides and receive input from the spinal cord, dorsal column nuclei, optic tectum and the cerebellum (Karten, 1967). However, it was later determined that the division receiving major spinal and dorsal column nuclei input lies ventral to the caudomedial regions of the central nucleus (Wild, 1995), and was termed the “preisthmic superficial area” (L. Puelles et al., 1994). A new “intercollicular area” was parsed out as a periventricular zone at the rostroventral end of the torus linked to vocalizations, unrelated to the intercollicular area in non-avian reptiles associated with somatosensation (L. Puelles et al., 1994). The preisthmic superficial area, as the name suggests, is topologically superficial to the central nucleus, consistent with the midbrain somatosensory area in other reptiles. The ventral location of the avian midbrain somatosensory area, rather than the lateral

location seen in other reptiles, is likely a result of being dragged inward during the massive invagination of the central nucleus (L. Puelles et al., 1994). The preisthmus superficial area also receives sparse projections from hindbrain cochlear nuclei, and the central nucleus in turn receives sparse projections from the spinal cord and dorsal column nuclei, suggesting convergence of somatosensory and auditory inputs in the torus semicircularis (Wild, 1995).

Similar to the torus in some reptiles, but to a greater extent, the torus semicircularis in mammals is caudally displaced to form a bulging “colliculus”, and the homologous structure is referred to as the inferior colliculus (Kuhlenbeck, 1975). The lateral cortex of the inferior colliculus (LCIC), synonymous with the external cortex (ECIC) or external nucleus (ICX), lies lateral to the central nucleus, and receives major ascending somatosensory input from the dorsal column nuclei, the spinal cord and the spinal trigeminal nucleus (e.g. Aitkin et al., 1981; Björkeland & Boivie, 1984; Coleman & Clerici, 1987; Morest & Oliver, 1984; RoBards et al., 1976; Zhou & Shore, 2006). We adopt the term LCIC following Oliver (2005), as it clearly denotes the lateral position, as opposed to other rostral, medial, and dorsal regions, and its superficial (“cortical”) affiliation. The LCIC receives sparse input from the brainstem cochlear nuclei (Aitkin et al., 1981; Coleman & Clerici, 1987; González-Hernández et al., 1996; Zhou & Shore, 2006), as well as projections from the central nucleus of the inferior colliculus and the auditory cortex (Bajo & Moore, 2005; Coleman & Clerici, 1987; Lesicko et al., 2016; Saldaña & Merchañ, 1992). Recent studies suggest that neurochemically distinct “modules” within the LCIC receive somatosensory input while the extramodular regions receive auditory input

(Dillingham et al., 2017; Lesicko et al., 2016). Cells that become the LCIC migrate from the embryonic ventricular zone (Repetto-Antoine & Meininger, 1982), and neurogenesis in the LCIC occurs earlier than in the central nucleus (Zeng et al., 2009). The location, connections and development of the LCIC of mammals share similarities with the somatosensory recipient area in reptiles. It is worth noting that an “intercollicular nucleus” has been described in mammals, located dorsal to the dorsal cortex of the inferior colliculus, ventral to the superior colliculus, medially bordering the periaqueductal grey and continuous with the medial edge of LCIC [using the definition in E. Puelles et al. (2011) and RoBards et al. (1976)], and it receives input from the ascending spinal pathways (Künzle, 1993; RoBards et al., 1976; Schroeder & Jane, 1976; Wiberg & Blomqvist, 1984). However, the presence of the intercollicular nucleus seems to vary between species (Schroeder & Jane, 1976). Its relationship with the LCIC is unclear. Additionally, while the ICX in birds shares the same nomenclature and occupies the same lateral location as the mammalian ICX, the avian ICX likely does not correspond to the vertebrate somatosensory midbrain in that 1) it does not receive somatosensory input (Wild, 1995), and 2) it is a part of the periventricular “toral nucleus”(L. Puelles et al., 1994), and its lateral location comparable to the mammalian ICX is a result of the inward protrusion of the avian torus.

A midbrain somatosensory area has also been found in anamniotes. Sharks have a lateral region in the dorsal midbrain that receives a spinal projection (Ebbesson & Hodde, 1981). In teleost fish, the external nucleus of the torus semicircularis is located lateral (i.e. superficial) to the lateral line recipient

ventrolateral nucleus and the auditory central nucleus, and receives somatosensory input, specifically from the spinal cord, sensory trigeminal nuclei, and the lateral funicular nucleus (Finger, 2000; Yamamoto et al., 2010). In frogs, all divisions of the torus semicircularis receives input from the dorsal column nuclei and from spinal and trigeminal afferents (Muñoz et al., 1995, 1997).

In contrast to most vertebrate species, the snake paratorus does not receive input from dorsal column nuclei, likely related to the small size of the dorsal column system in snakes (Ebbesson, 1969). Instead, a major input to the paratorus is the nucleus dorsalis myelencephali (DM) in the lateral caudal medulla (Schroeder, 1985). Teleost fish also have little to no dorsal column systems, and the lateral funicular nucleus, located in the lateral aspect of the caudal medulla, projects to the external nucleus of the torus semicircularis (Finger, 2000; Yamamoto et al., 2010). In amphibians, in addition to the connections from the dorsal column nuclei, the torus receives input from the lateral cervical nucleus (LCN) at the spinomedullary transition level (Muñoz et al., 1995, 1996, 1997, 1998), mirroring the location of the snake DM. The LCN was originally described in mammals and terminates mainly in the intercollicular nucleus and thalamus (Flink et al., 1983; Giesler et al., 1988; Menétrey et al., 1982; Wiberg et al., 1987) but also the LCIC (Wiberg et al., 1987).

The labelled neurons in DM and the cervical spinal grey in the present study reveal at least two spinotoral pathways in snakes. It is unclear whether the labelled fibers in the lateral funiculus beyond the cervical level originate in the axons of neurons in the spinal grey that were excluded from our histological material, or from an additional ascending pathway. In mammals, projections to the LCN arise in

spinocervical tract neurons located throughout the spinal cord, forming a disynaptic spinocervicomesencephalic pathway. A monosynaptic (direct) pathway relaying primary input via the LCN has not been reported in mammals. Additionally, there are neurons in the spinal gray that project directly to the midbrain via the spinomesencephalic tract in the lateral funiculus (reviewed in W. D. Willis & Coggeshall, 2004). In the dorsal funiculus, dorsal column nuclei neurons that project to the inferior colliculus form a distinct population to the ventral posterolateral thalamus projecting neurons (Lehnert et al., 2021; Turecek et al., 2022), and receive input from Pacinian corpuscles (Turecek et al., 2022). Pacinian corpuscles are low-threshold mechanoreceptors associated with perception of innocuous touch found in glabrous skin. In teleost fish, the major spinal ascending system to the torus is comprised of two monosynaptic pathways (relayed in the dorsal horn or lateral funicular nucleus) and a disynaptic pathway (relayed first in the dorsal horn and then in the lateral funicular nucleus; Finger, 2000; Yamamoto et al., 2010).

#### 4.2 Tuning of the paratorus

Auditory neurons in the snake paratorus responded to low frequency vibration. Thresholds of single auditory units were similar to, but slightly below, thresholds derived from vibration evoked thresholds (Han & Carr, 2023). The thresholds were higher than the thresholds for ball pythons and the rattlesnake (Christensen et al., 2012; Hartline, 1971b), which may be a result of interspecific variation or isoflurane anesthesia (Cederholm et al., 2012; Ruebhausen et al., 2012; Stronks et al., 2010; Thiele & Köppl, 2018). We are uncertain if auditory thresholds

for high frequencies exceeded somatic thresholds, since auditory units were not tested with the high stimulus levels used for somatic units.

Auditory units in the snake paratorus displayed a wide range of response properties, highlighting computational diversity in this structure. Few units in the paratorus phase-locked to the stimulus, similar to the torus of other species (e.g. Christianson & Peña, 2007; Kuwada et al., 1984). Response characteristics of neurons in the LCIC to auditory stimuli had only been investigated in mammals in a limited number of studies. Response patterns in the guinea pig LCIC include onset, primary-like (onset+sustained) and sustained (Jain & Shore, 2006; Syka et al., 2000), which can all be found in the snake paratorus. Additional response types in the snake paratorus such as late transient (delayed sustained), phasic erratic-latency (delayed onset) and on-off (irregular) have also been reported in the central nucleus of the inferior colliculus (Nuding et al., 1999; Pollak et al., 1978). Notably, neurons in the LCIC have extremely broad and irregular tuning to auditory input (Aitkin et al., 1975, 1981; Jain & Shore, 2006; Syka et al., 2000), responding to sound over a range of up to 6.5 octaves in cats (Aitkin et al., 1975, 1981). In contrast, the auditory neurons in the snake paratorus generally had V shaped tuning curves with distinct best frequencies, a characteristic of the central nucleus in birds and mammals (e.g. Aitkin et al., 1975; Ota et al., 2004; Wagner et al., 2002). Auditory input to the mammalian LCIC largely comes from the auditory cortex and ICC (Aitkin et al., 1981; Lesicko et al., 2016), whereas in the snake paratorus we found substantial projections from the auditory brainstem, which may explain these differences in frequency tuning. It is unclear which endorgans contribute to vibration responses in the paratorus auditory

units, since the nucleus angularis (cochlear), nucleus vestibularis ovalis (sacculus) and a potential third nucleus were labelled following dye injection in the paratorus. Nevertheless, although snakes lack tympanic middle ears, snakes and tympanate vertebrates share heterogeneous toral responses.

Paratorus somatic units in this study were characterized by an early transient response at onset, a cessation of activity and a period of sustained activity for frequencies at or below best frequency, and only phasic responses at onset for higher frequencies. The rate-level functions for different frequencies were not superimposable. In rattlesnake, evoked potential recordings in the somatic midbrain also had non-superimposable intensity-amplitude curves for different frequencies, and the intensity-latency curves for different frequencies had systematic differences. Hartline (1971a) suggested multiple peripheral receptors contributed to midbrain somatic responses; our single unit recordings support this hypothesis.

The somatic units in the snake paratorus were less sensitive than auditory units with best frequencies around 100 Hz. The only other study examining frequency tuning of somatic responses in the somatosensory midbrain was from Huey et al. (2024) in the LCIC of mice. LCIC neurons had best frequencies around 400 Hz to vibrations delivered to the hindlimbs, and the responses were absent in Ret cKO mice which lacked Pacinian corpuscles. Silencing of the LCIC abolished vibration detection in a behavioral assay. Note that the vibrational stimuli were calibrated to units of force (mN), which is not directly comparable to acceleration ( $\text{ms}^{-2}$ ) used in our study without information on the mechanical impedance of the vibrated object

(see Håkansson et al., 1985). Most LCIC neurons responded to both auditory and somatic stimuli (Aitkin et al., 1978; Huey et al., 2024; Zhou & Shore, 2006).

Pacinian corpuscles, which are a class of low-threshold mechanoreceptors found in glabrous skin on limbs that snakes lack, ascending to the LCIC via the dorsal column pathway (Turecek et al., 2022), a poorly developed pathway in snakes. On the other hand, the snake paratorus receives major projections from nucleus dorsalis myelencephali (DM), and the nucleus is similar to the lateral cervical nucleus in mammals in location and connections. Neurons in the mammalian lateral cervical nucleus are sensitive to hair movements along the body, including the trunk region, with large receptive fields (Djoughri et al., 1994; Giesler et al., 1979; Simone & Pubols, 1991). In cats, lateral cervical nucleus neurons projected only to the midbrain, only the thalamus, or both, and 92% of the midbrain projections neurons had receptive fields in the trunk (Djoughri et al., 1994). Spinocervical tract neurons, which relay peripheral input to lateral cervical nucleus neurons, have been shown to receive direct input from low-threshold mechanoreceptors in hairy skin (Abraira & Ginty, 2013; Brown et al., 1987). Considering the shared developmental origins of reptilian scales and mammalian hairs (Di-Poï & Milinkovitch, 2016), it is possible that homologous mechanoreceptors to hairy skin low threshold mechanoreceptors can be found in snakes and mediate the somatic aspect of vibration detection in the snake paratorus. Peripheral mechanoreceptors at the mid-thoracic region in Australian blacksnakes (*Pseudechis porphyriacus*) have similar frequency tuning to the western ratsnake paratorus somatic units (Proske, 1969b), and possibly corresponded to “end-button” receptors found in a histological study (Proske, 1969a). “End-buttons” have

simple anatomical structures and can be found in all regions of the body, in contrast to the complex “touch corpuscles” and “club corpuscles” only found in the head region (Proske, 1969a). Currently, the central connections of the “end-buttons” and their relationship to low threshold mechanoreceptors in mammalian hairs are yet to be explored.

#### 4.3 A hypothesis for the evolution of the unique snake torus

The small size of the central nucleus of the torus semicircularis in the western ratsnakes, and other Alethinophidian snakes (snakes other than blind snakes and thread snakes; Senn, 1969; personal observation), is likely associated with the loss of the tympanic middle ear. The central nucleus of the torus semicircularis is a midbrain center for processing ascending auditory input (Grothe et al., 2004). After the evolution of the tympanic middle ear, this ascending signal presumably became dominated by high-frequency airborne sound (Christensen-Dalsgaard & Carr, 2008; Christensen-Dalsgaard & Manley, 2013). The first snakes appeared after the evolution of the tympanic middle ear in lepidosaurs (Caldwell et al., 2015; Evans, 2016; Ford et al., 2021), and the lack of the tympanic middle ear in snakes is considered a secondary trait. The secondary loss of the tympanic middle ear during snake evolution would lead to the loss of high frequency input (Christensen-Dalsgaard & Manley, 2013; Manley, 2017; Walton et al., 2017), and likely led to the reduction in the size of the central nucleus of the torus semicircularis previously associated with hearing high-frequency airborne sound, since diminished or lost input leads to atrophy of central structures, as is evidenced in the reduction of tectal volume

and retinotectal pathways in the blind cave fish and the naked mole rat (Crish et al., 2006; Loomis et al., 2019; Voneida & Fish, 1984).

Alethinophidian snakes, from our personal observations and Senn (1969), have an exceptionally large midbrain somatosensory area (paratorus). Behavioral studies, which are a direct readout of function, have shown that the LCIC mediates vibration detection via an ascending somatosensory pathway in mice (Huey et al., 2024). While no behavioral experiments have been done in other vertebrate species to assess the functional role of the midbrain somatosensory area, the ample similarities in their physiology and connections suggest that its role in detecting low-frequency vibration perturbing the body may be congruent across vertebrates. Sensory specializations are usually reflected in an increase in size in the associated brain area, as the size of a neural structure reflects its processing capacity, i.e. principle of proper mass (Jerison, 1973). The large paratorus may reflect specialization in vibration detection mediated by somatosensory pathways.

An increase in the size of neural structures associated with somatosensation occurs widely among many fossorial vertebrates (e.g., star-nosed mole: Catania & Kaas, 1995; naked mole-rat: Henry et al., 2006). Evidently the midbrain somatosensory area of many extant fossorial lizards is intermediate between the snakes and tympanate lizards (Senn & Northcutt, 1973), suggesting that selection for increased computational capacity in the midbrain somatosensory area in fossorial lepidosaurs is a common trend. The functional implications of the large paratorus supports the proposed view that the most recent common ancestor of crown snakes was fossorial (Da Silva et al., 2018). The ancestral snake in the fossorial environment

likely faced low frequency seismic vibrations as a major source of sensory stimuli, which may have led to increased selective pressure for sensory specializations subserving vibration detection. However, the nature of the somatosensory specializations implied by a large paratorus is unclear.

The alternative explanation for a large paratorus is that neural input from the body has increased in snakes due to elongation of the trunk, because allometric studies have shown that snakes have greater body mass relative to brain mass compared to limbed lizards (Elizondo Lara et al., 2021). However, limbless lizards have elongated bodies and similar brain-body weight ratios to snakes (Elizondo Lara et al., 2021), and many tympanate limbless lizards do not have notable size gains in their midbrain somatosensory area (Senn & Northcutt, 1973; personal observations), suggesting that the increase in body surface area is not the determinant of an enlarged somatic midbrain.

An additional feature of the western ratsnake paratorus is its recipient of substantial input from hindbrain auditory nuclei, including cochlear nuclei and nucleus vestibularis ovalis. Although the first-order cochlear nuclei of the western ratsnake is reduced, we found that the sunbeam snake *Xenopeltis unicolor* has large inner ears and robust cochlear nuclei (Miller, 1980; personal observation), while maintaining an “extremely large” paratorus while the central nucleus “has absolutely no character of a nucleus but is a pure layer” (Senn, 1969; personal observation). This suggests that the ascending inner ear pathways in at least some Alethinophidian snakes are directed to the paratorus instead of forming connections with the central nucleus. In the western ratsnake, we were unable to find units that responded to

vibration in the central nucleus, which may reflect reduced input in the central nucleus from the auditory brainstem. In other vertebrates, the projection from the hindbrain cochlear nuclei to the midbrain somatosensory area, while sparse, have been widely reported (Aitkin et al., 1981; Coleman & Clerici, 1987; González-Hernández et al., 1996; Wild, 1995; Zhou & Shore, 2006), suggesting that the neuroanatomical framework for the auditory-paratorus connections is present in other vertebrates. However, we avoid generalizing the significant auditory projections in the paratorus to all Alethinophidian snakes, since the midbrain map of auditory and somatic response areas in rattlesnakes possibly matches the respective locations of the central toral nucleus and the paratorus (Hartline, 1971b).

Lastly, we point out that an increase in paratorus size is not shared among all extant snake species. Scolecophidian snakes, including blind snakes and thread snakes, are an infraorder of snakes traditionally considered sister to Alethinophidian snakes (Bellairs & Underwood, 1951; Miralles et al., 2018; Rieppel, 2012), although some recent studies have placed them as nest within Alethinophidia (Garberoglio et al., 2019; Palci & Caldwell, 2010; Strong et al., 2021). Regardless, Senn (1969) noted that many species have a central nucleus resembling that of lizards in form and of similar size to the paratorus. Since substrate vibrations can be sensed by the ear with or without eardrums (Capshaw et al., 2022; Womack et al., 2017; also see Chapter 3), it is possible that the connection between the ascending auditory pathway and the central nucleus is maintained by low frequency seismic vibration over the terrestrial-to-fossorial transition and further re-expanded during the evolution of the

Scolecophidian lineage, in contrast to some Alethinophdians, where the ascending auditory pathway formed additional connections with the paratorus.

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## Chapter 5: Conclusions and Future Directions

The detection of substrate vibration is often overlooked when discussing sensory biology. The experiments in this dissertation examined the neuroanatomical and/or physiological basis of vibration detection in several lepidosaur species and illustrated three possible routes that underly the vibration sense: cochlear, otolithic and somatosensory. Some findings, such as the diminished cochlear nuclei, may be specific to only certain snake species, while others may have more general applications, such as the hypertrophied midbrain somatosensory area found across Alethinophidian snakes, and saccular hearing implicated in all lepidosaurs. Although my research has addressed certain questions, it has also highlighted new areas that require further investigation, *in italics*.

I have described the first-order cochlear nuclei of the western ratsnake, which showed a remarkably small nucleus magnocellularis (NM) and a distinct, yet unimpressive, nucleus angularis (NA). However, I have observed considerable variation within snake species regarding the size of acoustic tubercle, more so than other reptile clades (personal observations; Miller, 1980), and the size of cochlear nuclei seems to be on the lower end of the spectrum for the western ratsnake. In most species we examined, the nuclei in the acoustic tubercle are often poorly differentiated, and separation of nuclear boundaries cannot be achieved with Nissl material alone. *A systematic survey using tract tracing would be required to document the diversity of the snake cochlear nuclei and examine the ecological or phylogenetic factors influencing cytoarchitecture.*

*The higher-order connections of the western ratsnake cochlear nuclei still remain to be mapped.* Chapter 4 showed that the paratorus receives projections from the superior olivary nucleus and the dorsal nucleus of the lateral lemniscus. Both structures have only been noted in schematical drawings of snake brains (Molenaar, 1976; ten Donkelaar & Nieuwenhuys, 1979), but their cytology and connections have not been previously described. In lizards, the superior olivary nucleus has two subnuclei (Foster & Hall, 1978; Tang et al., 2012; ten Donkelaar & de Boer-van Huizen, 1981; Yan et al., 2010), and the dorsal nucleus of the lateral lemniscus has three subdivisions (tokay gecko: Yan et al., 2010); it is unclear if similar divisions exist in the western ratsnake.

Additionally, injections in the paratorus labelled a novel structure in the acoustic tubercle of the hindbrain that is too medial and ventral to be a part of nucleus angularis (NA), and too rostral and medial to be nucleus vestibularis ovalis (VeO). *The input to this novel nucleus is unclear.* One hypothesis is that it represents the nucleus laminaris in snakes, homologous to the lateral edge of the nucleus laminaris in geckos, and that it receives bilateral input from nucleus magnocellularis. Support for this hypothesis is weak since the nucleus magnocellularis in the western ratsnake is extremely small, and the novel nucleus easily exceeds its size. Furthermore, no cells in the novel nucleus have the typical vertically oriented, bitufted neuronal architecture of nucleus laminaris in other species. An alternative hypothesis is that this novel nucleus receives input from another inner ear end-organ, which may be resolved with additional tract tracing studies. In the gecko and possibly other tympanate lizards, NA and VeO have clear spatial separation, whereas NA, VeO, and

the novel nucleus have indistinct borders in the western ratsnake. Considering that the vibrogram for the snake auditory system (=originating from the inner ear) is U-shaped with only one best frequency (Christensen et al., 2012; Han & Carr, 2023; Hartline, 1971b), it is interesting to propose that Hebb's law links these three seemingly redundant nuclei and their roles in vibration detection of snakes. Conversely, we note that vibration sensitivity for the auditory system in different snakes has only been assessed along the dorsoventral axis (Christensen et al., 2012; Han & Carr, 2023; Hartline, 1971b, 1971a); *it is possible input from different auditory endorgans encode sensitivity along different axes.*

In a similar vein, we only characterized the frequency tuning of VeO neurons in the tokay gecko using a dorsal-ventral vibration. In frogs and teleost fish, which have saccular hearing, the saccular macula is curved, and the array of saccular hair cells have different orientations that encode an array of sound sources, not restricted to one direction on the vertical plane (Cazin & Lannou, 1976; Edds-Walton & Popper, 1995; Hetherington et al., 1986; Ladich & Schulz-Mirbach, 2016). Thus, different hair cells populations are activated by vibrations in different directions, and primary input from the saccule conveys information from many directions. *Currently, the hair cell orientation on the saccular macula of lepidosaurs is unknown.* Resolving the orientations of saccular hair cells in snakes may also address the aforementioned “redundancy” of different inner ear pathways for vibration detection.

*The connections of the paratorus have not been mapped beyond the level of spinocervical junction.* The inputs to the nucleus dorsalis myelencephali, which projects to the paratorus, can be uncovered with tracer injections in the dorsal root

ganglia and the nucleus itself. Additionally, it is possible to isolate the mechanoreceptors projecting to paratorus via antidromic activation and collision testing, and determine their fine structure with immunostaining.

The somatosensory midbrain seems to subserve vibration detection in vertebrates. The large size of the snake paratorus indicates specialization from an anatomical perspective, however my results failed to show its functional purpose. The nucleus dorsalis myelencephali sends bilateral projection to the paratorus, and the paratorus is connected with the contralateral homologue via commissural fibers. Combining reports of somatotopy in the midbrain somatosensory area in other vertebrates (Björkeland & Boivie, 1984; Wiberg et al., 1987; Wild, 1995), *it is enticing to postulate that snakes could use the somatic system for vibration localization*. Behavioral evidence in the horned viper (*Cerastes cerastes*) indicates that snake can localize the source of vibration (B. A. Young & Morain, 2002). While B.A. Young & Morain (2002) concluded that the horned viper uses vibrational cues to locate prey, they did not investigate which mechanoreceptive sensory systems were involved. Considering that there is poor anatomical support for ITD computation in the auditory brainstem - both NM and NL are small in the western ratsnake - somatosensory pathways may provide an alternative route for vibration localization. However, as with any untested hypothesis, this theory requires further systematic evaluation.

*The multitude of pathways for vibration detection in lepidosaurs hints at roles beyond predator-prey interactions, such as intraspecific communication.* Chameleons have been shown to produce low frequency biotremors for communication with

conspecifics (Barnett et al., 1999; Denny et al., 2023b, 2023a; Tegge et al., 2020), although the underlying sensory modality is unknown, and research on the anatomy and physiology of the vibration-sensing pathways is needed. Among snakes, low frequency body vibrations are often produced, especially males, during courtship and copulation (e.g. Noble, 1937; Senter et al., 2014; Shetty & Shine, 2002). Some of these species, like *Xenopeltis*, are semi-fossorial and inhabit muddy soil through which these body vibrations could potentially propagate without direct contact (B. A. Young, 2003). If and how these signals are processed via the mechanisms described in this dissertation is unclear.

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