COMPARATIVE TOPICS IN VERTEBRATE MECHANORECEPTION WITH A SPECIAL FOCUS ON THE CROCODILIANS

By

Duncan B. Leitch

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Approved:

Professor Kenneth C. Catania

Professor Patrick Abbot

Professor Jon H. Kaas

Professor Mark T. Wallace

DEDICATION

For my father, Paul, and mother, Soledad, who always encouraged my fascination with nature and all of its inhabitants

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LIST OF ABBREVIATIONS

Aud	Auditory-responsive cortex
BS	Brainstem
C	Caudal
Cap	Capillary
CO	Cytochrome oxidase
CoV	Coefficient of variance
DPR	Domed pressure receptor
DVR	Dorsal ventricular ridge
En LamC	Encapsulated lamellated corpuscle
EtOH	Ethanol
FBR	Free branched receptor
gV	Trigeminal ganglion
IR	Infrared
ISI	Interspike interval
ker	β-keratinocyte
KOH	Potassium hydroxide
L	Lateral
LamC	Lamelleated corpuscle
ISO	Integumentary sensory organ
M	Medial
Mel	Melanocyte
mya	Million year ago
NMotV	Trigeminal motor nucleus
OB	Olfactory bulb
OT	Optic tectum
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PNS	Perineural sheath
PrV	Principal trigeminal nucleus
R	Rostral
RA	Rapidly adapting
RAx	Branched receptor axons
S1	Primary somatosensory cortex
S2	Secondary somatosensory cortex
SA	Slowly adapting
StC	Stratum corneum
StL	Stratum lucidum
StS	Stratum spinosum
SVL	Snout to vent length
Tel	Telencephalon
TrSpMes	Spino-mesencephalic tract

TS	Tentacled snake
SD	Standard deviation
Sp	Spinal cord
V1	Primary visual cortex
V1	Ophthalmic branch of the trigeminal nerve
V2	Maxillary branch of the trigeminal nerve
V3	Mandibular branch of the trigeminal nerve
VF	von Frey hair

CHAPTER I

Introduction

"How doth the little crocodile
Improve his shining tail,
And pour the waters of the Nile
On every golden scale!

How cheerfully he seems to grin,
How neatly spreads his claws,
And welcomes little fishes in
With gently smiling jaws!"

Lewis Carroll, 1865, *Alice's Adventures in Wonderland*

A fascination with the behaviors of animals seems to be a unique quality that connects most human beings, regardless of age or background. Indeed, from observing animals interacting with their environments and with one another, we seem to learn more about ourselves. From the singing of a bird to the flight of a bat, behaviors emerge smoothly and seemingly effortlessly, giving no hint to the vast network of interconnected nerve cells which functions simultaneously in the background to produce these coordinated phenomena. These dynamic patterns of neurons firing together, driving the activity of other neurons, and inhibiting the action of others, produce the repertoire of behaviors noted in the worlds of animals. Despite decades of research directed towards explanations of the neural control underlying behavior, our understanding of such systems is incomplete and remains one of the fundamental questions of neurobiology.

The roots of neuroethology are well summarized by Camhi who described that “the reason nervous systems evolved in the first place was to produce behavior, and to do so the out-of-doors, under the full blare of nature’s physical forces” (Camhi, 1984), a perspective that inherently requires an appreciation of ethology. The knowledge derived from the synthesis of neurobiological and ethological perspectives is greater than when one is arbitrarily limited to the methodical and intellectual approaches from a single “field.”

The countless examples of the utility of this approach in regards to understanding the neuronal mechanisms underlying novel behaviors or specializations run the gamut of the animal kingdom and have revealed much about the fundamental properties of the nervous system. These include studies of the following, to mention only a few: A) Examinations of the brain structures associated with vocal learning and production in song and non-song birds have shed light on the morphological and physiological plasticity of the nervous system (Marler, 1990; Nottebohm, 1970). From a broader, comparative perspective, examinations of a variety of birds, mammals, and reptiles have shown increased expression of the first gene linked to human speech within specific brain nuclei related to song learning in species of birds capable of vocal learning (Haesler et al., 2004; Rochefort et al., 2007). B) Analyses of the electrosensory system of fish, enabling species to locate weak electric fields in the water, have demonstrated their utility in studies of receptor structure (Claudio et al., 1983; Hershey et al., 1983), ion channels (Lu and Fishman, 1995; Stoddard et al., 2006), and neural circuitry (Heiligenberg, 1989; Rose, 2004; Zakon, 1993). The widespread but spotty presence of this seemingly-exotic sensory modality in a number of vertebrates, including all classes of fishes and some orders of amphibians and mammals, suggests that electroreception is an ancestral trait that has "re-evolved" multiple times (Liebeskind et al., 2011; Zakon, 2002; Zakon, 2012). C) Work examining the neural substrates underlying attachment in the monogamous prairie vole and the related polygamous montane vole (Carter et al., 1995; Carter and Getz, 1993; Carter et al., 1980) has been fruitful in the realm of neuroendocrinology and has identified the neuropeptides oxytocin and vasopressin as playing key roles in the mammalian pathways associated with social behavior (Young et al., 2001). Mesolimbic dopamine pathways associated with reward learning and reinforcement are modulated in response to fluctuating levels of neuropeptide receptors (Aragona et al., 2003; Wang et al., 1999) and such examples provide interesting implications for the neurobiology of pair bond formation (Young and Wang, 2004). D) A large body of literature exists on the neural mechanisms of the auditory and visual systems of barn owls. Following early behavioral experiments focused on the use of hearing in prey localization by owls (Payne, 1971), researchers controlling the location of sound stimuli relative to the owl's head demonstrated the bird's use of sound intensity and timing differences in each ear to provide information about the vertical and horizontal position of the sound source (Knudsen and Konishi, 1979; Moiseff and Konishi, 1981). Further work has identified a morphologically- specialized array of cells in the laminar nucleus that essentially creates a "computational map" that corresponds to particular locations in auditory space, based on the timing differences of a sound arriving at each of the owl's ears (Carr and Konishi, 1990). The concept of such a map has intriguing implications for understanding the neural circuitry underlying our own perception of sound.

It is in embracing this comparative spirit that I have chosen to examine the vast topic of how nervous systems direct animal behavior by subdividing the challenge into more manageable questions suitable to careful study. Much of my attention has focused on the somatosensory system – an extensive grouping of senses that includes responses to touch, pain, and muscle position that enables a particular organism to respond to a broad range of physical stimuli such as pressure, stretch, or thermal changes. As a sensory modality thought to have existed in invertebrate ancestors, it is present and widely studied in a variety of relatively simple "model" organisms such as *Drosophila (*Walker et al., 2000) and *Caenorhabditis elegans* (Syntichaki and Tavernarakis, 2004) to mammalian systems such as the vibrissae of rodents. Currently, comprehensive genomic screens for candidate genes encoding protein channels involved in mechanotransduction are being performed in both mammalian (Gerhold et al., 2013; Gracheva et al., 2011) and non-mammalian systems (Gracheva et al., 2010) in attempts to uncover the molecular basis of somatosensory systems; the recognition that these assays have identified genes with mammalian homologs underscores the evolutionary conservation and importance of somatosensory signaling mechanisms (Lumpkin and Bautista, 2005). In comparison to other more widely-studied sensory modalities, particularly the visual system, our understanding of the transduction mechanisms responsible for the numerous percepts that fall under the "somatosensory" umbrella is still in its infancy.

Apart from elucidating the signal transduction pathways mediating mechanoreception, specialization of vertebrate body morphology and peripheral nervous systems related to somatosensation have been well appreciated (Johnson and Reed, 2008). From the extensions of the body surface as in the case of the elaborate vibrissae of many mammals or the unique, whisker-like feathers of some birds (Cunningham et al., 2011) to densely-innervated touch spots with groupings of mechanosensitive receptor endings as in the case of the glabrous skin of the mammalian fingertips and lips, the somatosensory system is capable of detecting objects and events at a distance as well those which come into direct contact with the skin. This information is of vital importance in detecting prey and preventing thermal and mechanical damage to the body, among other tactile-related tasks (Julius and Basbaum, 2001). Different aspects of these modalities are conveyed via specific specialized end-organs of the receptors (e.g., Merkel cells for texture discrimination and Pacinian corpuscles for vibration), and these are distributed non-uniformly across the body surface and in the follicle complexes of vibrissae. Indeed, a great deal of our insight into vertebrate somatosensation and its nervous system correlates has arisen through the adoption of a comparative approach in examinations of species with distinctive or unfamiliar peripheral adaptations. Among these diverse species, the naked mole-rat (*Hetercephalus glaber*) has an array of non-facial body hairs (Crish et al., 2003) and large incisors, both of which are represented in the cortical regions processing somatosensation (Catania and Remple, 2002) and aid in navigating and feeding in subterranean tunnels. A similar change in the central nervous system as a reflection of the periphery can be seen in the "barrel" system of many rodents in which neural representations of each individual mystacial whisker can be readily visually identified at various relays of the trigeminal system including the neocortex (Woolsey and Van der Loos, 1970) and brainstem nuclei. Perhaps even more unusual are the somatosensory specializations of the pressure-sensitive push rods and electroreceptive duct gland receptors (both innervated by the trigeminal nerve) which are organized into striped bands on the bill of the platypus (*Ornithorhynchus anatinus*) (Pettigrew, 1999). Although the peripheral organs are adjacent to one another, they form physiologically and visibly distinct striations on the surface of the cortex (Krubitzer et al., 1995; Manger et al., 1996). Presumably, this allows the platypus to locate voltage-generating prey and subsequently make tactile contact with the bill, conferring a greater success rate in underwater predation.

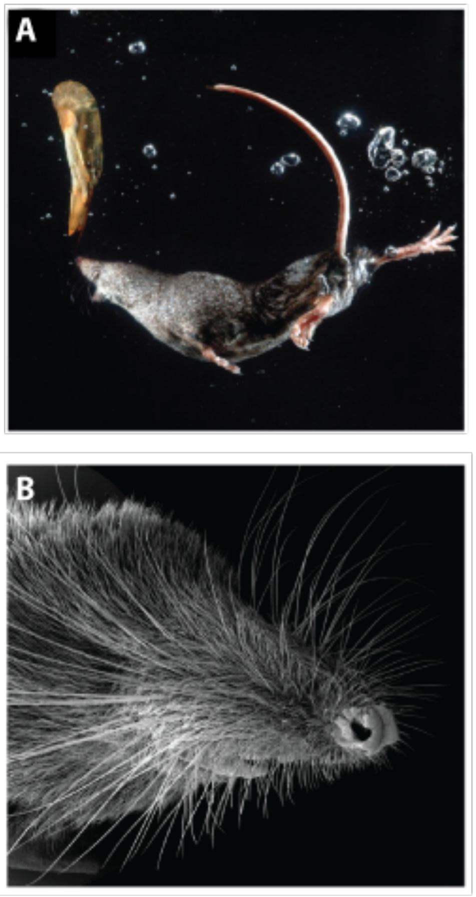
This project has been united by observations of the adaptations of the periphery related to tactile discrimination as well examinations of the central nervous system. The sensory systems, and in particular, somatosensation have offered the unique opportunity to look at the convergent mechanisms that a variety of taxa have developed in order to sense information vital to survival. What challenges does an animal face in detecting in somatosensory or mechanical stimuli in terrestrial and aquatic environments? What functions are mediated by novel peripheral nervous system adaptations? How are these "specializations" reflected in the nervous system of the animal? The process of searching for an answer to such questions has inherently relied on an appreciation of each organism's unique behaviors of and consideration of how similar mechanosensory solutions could have evolved among divergent groups of animals.

Somatosensation in Water Shrews

Members of the historical order Insectivora (now classified as Eulipotyphla) have long interested comparative and evolutionary neurobiologists who have pondered the anatomy of ancestral mammalian brains (Ebner, 1969; Regidor and Divac, 1992; Stephan et al., 1991). In particular, moles (family Talpidae) and shrews (family Soricidae) have unique body and brain morphologies that appear similar to Laurasiatherian mammal fossils, and endocasts from such fossil specimens have revealed small lissencephalic brains with relatively little neocortex – the 6-layered structure appreciated as the hallmark of the mammalian brain (Gebo, 2004; Kielan-Jaworowska et al., 2004). These central nervous system configurations are shared with many extant shrews and moles. In comparison to extensive morphological investigations that have been undertaken on the skeletal system and musculature of moles and shrews (Chapman, 1919; Cramer, 1951; Dobson, 1882; Freeman, 1886; Sánchez-Villagra et al., 2006), examinations of the organization of the neocortex and peripheral nervous system have been relatively few and, historically, have yielded conflicting results. Consistent with the idea of eulipotyphlans being "primitive" with relatively undifferentiated sensory areas of cortex, Lende recorded broad, indistinct overlapping areas dedicated to the processing of somatosensory, visual, and auditory modalities in the hedgehog (family Erinaceidae) (Lende and Sadler, 1967). More recently, systematic *in vivo* electrophysiological investigations of the neocortex of soricine shrews (Catania et al., 1999; Naumann et al., 2012; Roth-Alpermann et al., 2010), talpid moles (Catania, 2000), and hedgehogs (Catania et al., 2000) have revealed distinct primary sensory areas which are found adjacent to one another yet not overlapping, suggesting that the notion of these unique mammals as having poorly defined, unorganized central nervous systems appears erroneous.

As a species, the American water shrew (*Sorex palustris*) displays a number of impressive behavioral traits, especially in light of their diminutive body size (12-15 g). As the smallest homeothermic mammalian divers (Calder, 1969), they are capable of rapidly locating and attacking prey in both aquatic and terrestrial environments throughout the northern United States and across Canada (Fig. 1A). These attacking behaviors can be elicited by subtle water movements on the impressive array of mystacial vibrissae (Fig. 1B), stimuli likely to be encountered while foraging for fish and aquatic invertebrates (Catania et al., 2008; Conaway, 1952). Such whisker-mediated aquatic prey localization behaviors are similar in many respects to those seen in the pinniped harbour seals (Phocidae) and California sea lions (Otariidae) in response to hydrodynamic wakes (Dehnhardt et al., 1998; Dehnhardt et al., 2001; Glaser et al., 2011). Perhaps even more unusual is the observation of water shrews (and star-nosed moles, *Condylura cristata*) exhaling and re-inhaling air bubbles through their nostrils to provide olfactory cues while foraging (Catania, 2006). Other research groups have recorded ultrasonic vocalizations from some species of shrews and have proposed that shrews may use a form of echolocation to navigate through subterranean tunnels (Gould et al., 1964; Siemers et al., 2009), similar to the well-established roles of echolocation in microchiropteran bats and marine mammals. With respect to the diverse repertoire of sensory behaviors in both aquatic and terrestrial environments, the elaborate peripheral array of facial vibrissae, and their unique yet debated place within mammalian evolution, the “primitive” water shrew offered an opportunity to examine peripheral and central nervous system organization.

Fig. 1 American water shrews (*Sorex palustris*) are agile predators both in aquatic and terrestrial settings. **A.** Photograph of a water shrew demonstrating its diving capabilities in pursuit of a crayfish. **B.** Scanning electron micrograph of the elaborate array of mystacial vibrissae which are used in the detection of water movements and shape-related information. The copyright to 1A is held by Kenneth C. Catania; modified from Catania et al., 2008.


A
B

Somatosensation in Tentacled Snakes

The curious morphology of the tentacled snake (*Erpeton tentaculatus*) immediately begs the question of the function of the distinct appendages projecting from the rostral regions of the head (Fig. 2). Found in the murky rice paddies and streams of Thailand, Cambodia, and Vietnam, they are adapted to live almost exclusively in the water and are thought to feed solely on small fish. Previous behavioral work had uncovered the unique foraging strategies used by tentacled snakes, exploiting the hard-wired reflexive C-start escape responses of fish by making subtle feinting movements with their bodies as the fish swam into an area near the head and trunk in a clear example of the "rare enemy" effect (Catania, 2009). However, the specific function of the appendages, a question that had puzzled herpetologists and anatomists since the late 19th century, remained elusive. In the past, suggested functions included their usage in tactile discrimination of aquatic prey (Bellairs, 1970; Gunther, 1864) yet Hahn stated unequivocally that the tentacles served "no major sensory function" due to their complete lack of sensory tissues (Hahn, 1973). He also disagreed with any possible luring function of the tentacles, as found in the worm-like lingual lure of the alligator snapping turtle *(Machrochelys temminckii*) (Spindel et al., 1987) or tails of juvenile cottonmouth (Agkistrodon sp.) snakes (Wharton, 1960). Together with its unusual body poses and coloration he believed that the main function of the appendages was in crypsis (Hahn, 1973).

Fig. 2. The tentacled snake (*Erpeton tentaculatus*) is the single known species of its genera in the family Colubridae. It is notable for its distinctive appendages extending from the rostrum as well as its unusual fish-catching behaviors. A juvenile snake is extending from a submersed log and adopting the J-shaped neck pattern used in performing predatory strikes. Photograph from KCC.

Taking into account their novel aquatic predatory behaviors and anatomical similarities to other species with mechanosensory facial elaborations (for star-nosed moles see (Catania, 1995); for fish with tactile barbels see (Marui and Caprio, 1982)) in comparison to other snakes as well as vertebrates more broadly, the tentacled snakes were intriguing. With the opportunity to examine the relationship between peripheral anatomy and central nervous systems' topographical "maps" of representation in comparison to similar electrophysiological preparations from species as different as green iguanas (Stein and Gaither, 1981; Stein and Gaither, 1983) to cats (Stein et al., 1976), I was eager to contribute to projects involving these unique reptiles.

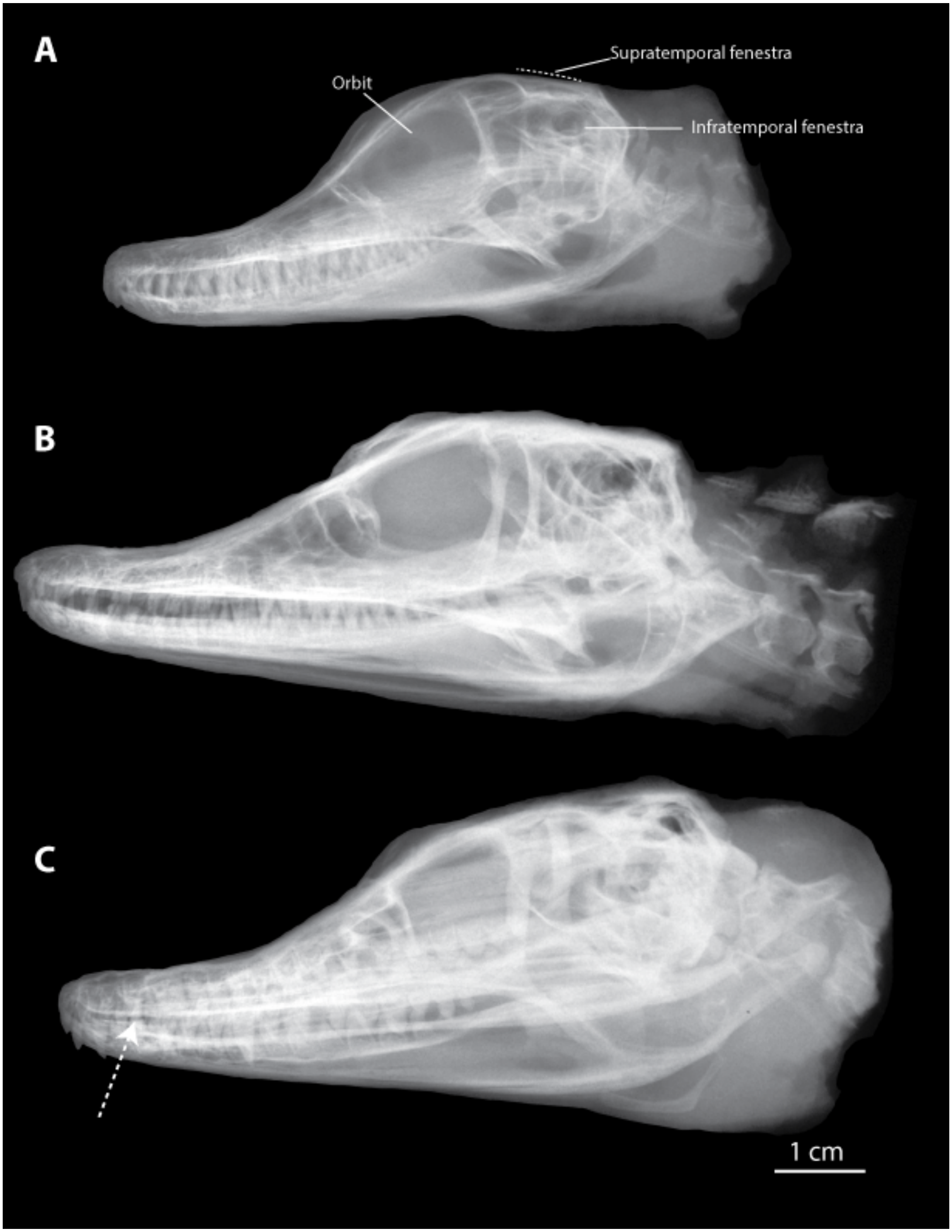
Somatosensation in Crocodilians

The idea of "primitive" beasts capable of overpowering and consuming humans and other large mammals is horrifying and fascinating in the same breath. Such is the case with the crocodilians and a handful of other vertebrates. Not only can they injure us, but they also can eat us. They remain as living reminders in a world of iPhones and satellite television that powerful forces, tens of millions of years in the making, have shaped creatures capable of quickly pulling us out of our comfort zones and transferring us back to a time when the idea of being pursued for our lives was plausible, if not likely.

As I write this, there are no fewer than three television series that prominently feature crocodilians that can be watched every night of the week - often multiple times on the same night (2010a; 2010b; 2012). The world's longest captured crocodilian, Lolong, a Saltwater crocodile (*Crocodylus porosus*) more than 6 meters in length was found just last year (Dell'Amore, 2011). Everyday a news story appears about a small American alligator found in a Long Island parking lot (Buiso, 2012), a child getting attacked by a Nile crocodile in remote Zambia (Ntandaa, 2013), or an alligator literally inhabiting the sewers beneath a well-populated city (Helsel, 2010). Humans cannot help but be fascinated by the mythologized crocodiles and their simple existence blurs the lines between fantasy and reality with their ancient, dinosaur-like appearances.

The 23 extant species of crocodilians remain of exceptional interest not only to the general public but to diverse groups of researchers including paleontologists, paleobiologists, anatomists, and physiologists, with the tempting offer of a glimpse into the physiology and behavior of dinosaurs and other extinct vertebrate lineages (Brazaitis and Watanabe, 2011), with their ancestors tracing back to the Late Triassic, approximately 225 million years ago. They are part of a diverse group of vertebrates known as the archosaurs ("ruling reptiles", Cope 1869) and can be distinguished from other amniotes by the presence of two large openings on each side of their diapsid skulls (the temporal fenestra) compared to the single-holed anapsid skulls in turtle-like ancestors (Fig. 3A). Although the only extant archosaurs besides crocodilians are modern birds, members of the crown group Archosauria also included the dinosaurs and several groups restricted to the Mesozoic. Multiple examinations of large-scale phylogenies of Archosauria have arrived at conflicting conclusions regarding which members of the crocodylomorph clade (Crurotarsi) are most basal; however, this confusion may be a function of the synapomorphies being characterized in limited taxa.

Fig. 3. X-rays of the skulls of three extant juvenile crocodilians. **A.** A juvenile American alligator (*Alligator mississippiensis*) shows the hallmarks of the diapsid skull with two openings, the supratemporal and infratemporal fenestra, posterior to the orbit of the eye. **B.** The skull of another alligatorid, the smooth-fronted caiman (*Paleosuchus trigonatus*). Note the prominent armored nuchal scales on the dorsal surface of the neck. **C.** The skull of a crocodilid, the Nile crocodile (*Crocodylus niloticus*) shows a more tapered appearance of the bones anterior to the orbit as well as the distinctive notch in the maxilla (arrow) that makes the 4th mandibular tooth visible in most crocodiles when the mouth is closed.


A
Orbit
Supratemporal fenestra
Infratemporal fenestra
B
C
1 cm

Despite challenges to archosaurian phylogeny, there is consensus about the presence of enormous crocodile-like reptiles during the early Cretaceous (approximately 73-80 mya). These include *Deinosuchus*, the so-called "terror crocodile," which is thought to have been up to 12 m in length and possess a mass of more than 8.5 metric tons (Erickson and Brochu, 1999; Schwimmer, 2002). This species was relatively recently dethroned as the largest crocodilian ancestor by reconstructions of *Sarcosuchus* (the "Supercroc") which is estimated to have a body length of 12.2 m or roughly the length of a city bus and possess a skull as long as an average human's height (Sereno et al., 2001). Interestingly, these formidable reptiles were remarkably similar in general body and skull morphology to their present day counterparts and are thought to have attained such tremendous sizes through an extended period of development prior to maturity as opposed to having an unusually high growth rate (Erickson and Brochu, 1999).

As an order of reptiles that has adapted to most tropical, semi-aquatic environments across the world, modern crocodilians are keystone predators, shaping their ecosystems with their presence and behaviors. They are divided into three families: 1) Alligatoridae, typified by the American alligator and South American caiman species; 2) Crocodylidae, as represented by the Nile crocodile and Saltwater crocodile; 3) Gavialidae, the critically-threatened Indian crocodilian species notable for its elongated, slender snout adapted for fish-catching (Busbey, 1995; Thorbjarnarson, 1990). Indeed the rostral shape of the jaws can be used to distinguish between many crocodilian taxa and individual species, dividing the short-snouted (brevirostrine) alligatorid and crocodilids with robust snouts capable of overwhelming large terrestrial prey from the long-snouted (longirostrine) species which consume mostly fish (McHenry et al., 2006; Walmsley et al., 2013)(Fig. 4). In side profile, the heads of crocodilians often appear "toothier," as the fourth mandibular tooth fits within a notch of the maxilla and remains visible when the jaws are closed (Fig. 3C). On the other hand, in the alligatorids, the mandibular teeth fit into sockets between the maxillary teeth, obscuring the appearance of the teeth of the lower jaw when the mouth is closed (Huchzermeyer, 2003).

Another notable distinction between the crocodilian families lies in their distributions of small (<1 mm^2), darkly-pigmented, raised domes on the skin surface. Known by as integumentary sensory organs (ISOs) (Brazaitis, 1987), they have been appreciated as a means of discriminating species of crocodilian skins. They are present on the jaws near the teeth of all crocodilian species, including the alligatorids, (Fig. 5) but are distributed more widely on the crocodylids and gavialids, typically found as one to three per scale on the rest of the ventral body surface. Although their presence had been noted since the early 20th century (von Wettstein, 1937), elucidating the specific functions and sensations mediated by the ISOs remained challenging. Speculative sensory modalities directed by ISOs ranged from functioning as "salinity detectors," particularly in the case of the post-cranial receptors found on crocodiles (Jackson and Brooks, 2007; Jackson et al., 1996) to playing a role in other more exotic systems such as sensitivity to the earth's magnetic fields (Rodda, 1984) or electroreception (Bullock, 1999). Despite these sensory systems' seemingly random appearance among vertebrate lineages, the notion that the ISOs might function in a unique capacity seemed to have some credence, stemming from crocodilians' semi-aquatic lifestyle, extensive navigation

Fig. 4. Comparisons of dorsal views of the heads of extant crocodilians demonstrate the diversity of snout morphologies. **A.** Among the most-broadly snouted is the American alligator (*Alligator mississippiensis*) with its rounded, shovel-shaped head. **B.** The smooth-fronted caiman (*Paleosuchus trigonatus*), though still an alligatorid, has a moderately tapered snout. **C.** The Siamese crocodile (*Crocodylus siamensis*) exhibits an even sharper snout, in keeping with its largely fish-based diets supplemented with small mammals, reptiles, and amphibians. **D.** The African slender-snouted crocodile (*C. cataphractus*) possesses an even sharper snout and is more reliant on fish that most other crocodile species. Although not shown, the most elongated snout among the living crocodilians belongs to the sole member of the Gavialidae family, the gharial (*Gavialis gangeticus*).

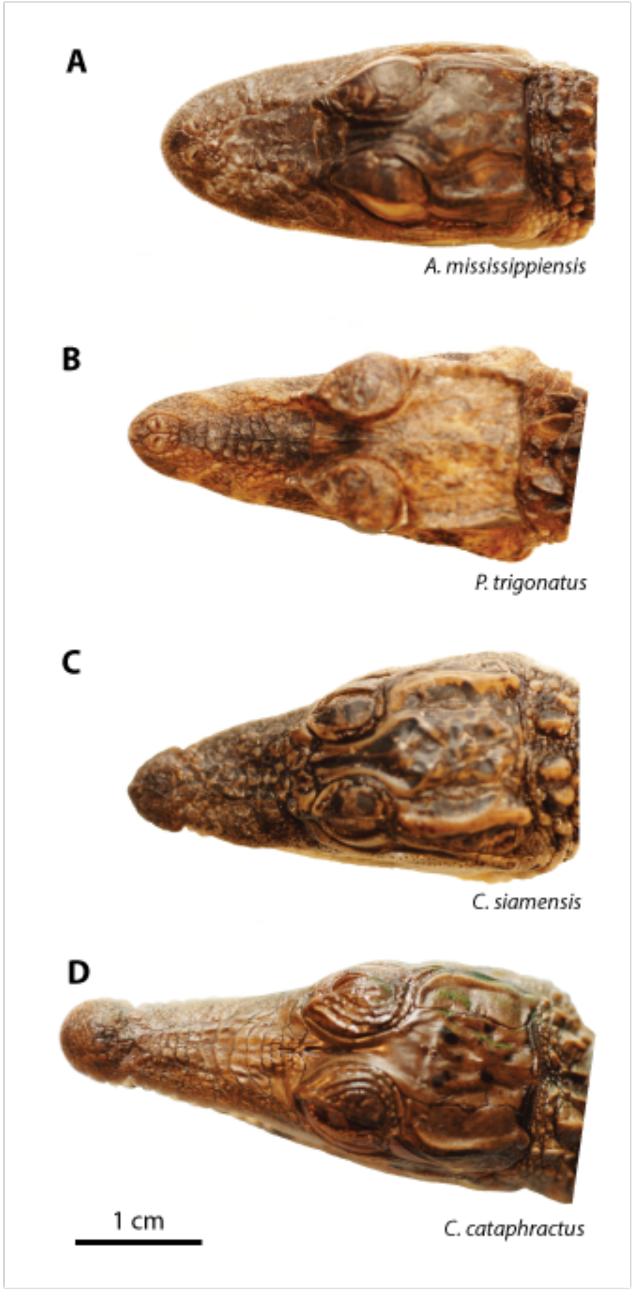

A
A. mississippiensis
B
P. trigonatus
C
C. siamensis
D
1 cm
C. cataphractus

Fig. 5. Portrait of a juvenile American alligator (*A. mississippiensis*). The upper and lower jaws of all crocodilians are covered in thousands of integumentary sensory organs (ISOs) which are visible in this photograph as circular black dots against the yellow skin of the face. They are particularly noticeable and densest in locations adjacent to the teeth. ISOs are found on the body scales of members of the families Crocodilidae and Gavialiadae, but these distributions are absent in the Alligatoridae

abilities, and tremendous predatory abilities. Adopting an admirable neuroethological approach, Soares (Soares, 2002) made headway in discerning the function of the ISOs, demonstrating that those of juvenile American alligators (*Alligator mississippiensis*) could mediate orienting responses to the water surface ripples created by a single falling droplet of water with auditory and visual cues blocked. In the process, she also coined a new terminology for the ISOs, calling them domed pressure receptors or DPRs. Yet, many questions still remained. Did the ISOs of the body of crocodiles work in a similar fashion to those of the face of alligators? How sensitive were the ISOs? What behaviors might they mediate following the animal's initial movements towards the small water splash?

With an eye towards appreciating the naturalistic behaviors of crocodilians, I adopted a three-tiered approach in answering these questions. This involved examining the microanatomy of individual cranial and body ISOs, visualizing the larger patterns of innervation from the central and peripheral nervous system to ISO-covered skin surfaces, and recording directly from the afferents of the ISOs. In this manner, I hoped to have formed a more comprehensive view of the sensory functions of the ISOs and the behaviors to which they might contribute.

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CHAPTER II

Chemoarchitecture of Layer 4 Isocortex in the American Water Shrew (*S. palustris*)

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Abstract

We examined the chemoarchitecture of layer 4 isocortex and the number of myelinated nerve fibers for selected cranial nerves in the American water shrew (*Sorex palustris*). This study took advantage of the opportunity to examine juvenile brain tissue, which often reveals the most distinctive cortical modules related to different sensory representations. Flattened cortical sections were processed for the metabolic enzyme cytochrome oxidase (CO), revealing a number of modules and septa. Subdivisions related to sensory representations were tentatively identified by performing microelectrode recordings in a single adult shrew in this study, combined with microelectrode recordings and anatomical findings from a previous investigation. Taken together, these results suggest that characteristic chemoarchitectonic borders in shrew neocortex can be used to delineate and quantify cortical areas. The most obvious subdivisions in the water shrew include a relatively small primary visual cortex that responded to visual stimuli, a larger vibrissae representation in S1, and a prominent representation of oral structures apparent in more rostral-lateral cortex. A presumptive auditory area was located in far caudal cortex. These findings for the cortex are consistent with counts from optic, auditory, and trigeminal nerves, suggesting somatosensory inputs dominate the shrew's senses whereas visual and auditory inputs play a small role in navigation and in finding prey. More generally, we find that shrews share unusual features of cortical organization with moles, supporting their close taxonomic relationship.

Introduction

Comparative and evolutionary neurobiologists have often studied members of the historical order Insectivora with the goal of understanding potential configurations of ancestral brains (Ebner, 1969; Lende, 1969; Kaas et al., 1970; Valverde and Facal-Valverde, 1986; Glezer et al., 1988; Michaloudi et al., 1988; Stephan et al., 1991; Regidor and Divac, 1992). This approach has been taken in part because ancestral mammals had small brains with little neocortex (Kielan-Jaworowska et al., 2004; Gebo, 2004) and some insectivores, particularly shrews (family Soricidae), resemble small-bodied ancestral mammals based on fossil evidence. Despite this long-standing interest and the impression that modern Soricine shrews may retain primitive characters, few studies have examined the details of their cortical organization (Catania et al., 1999; Roth-Alpermann et al., 2010). Beyond the historical (though questionable) interest in shrews as representatives of ancestral mammals, shrews are of general interest for comparative studies aimed at reconstructing the most likely configurations of ancestral brains based on the distribution of characters across a wide range of mammalian lineages (Northcutt and Kaas, 1995; Krubitzer, 1998; 2009; Kaas, 2005). On a smaller scale, the taxonomic relationships of shrews, moles (Talpidae), hedgehogs (Erinaceidae) and other insectivores remain uncertain (Symonds, 2005) and shared derived features of brain organization may provide additional traits for establishing phylogenies. Shrews also represent some of the smallest mammalian species, and thus can provide clues to how the smallest sheets of 6-layered isocortex may be uniquely organized – i.e., are there constraints on cortical organization based on size? Finally, historical views of insectivores having poorly organized brains and correspondingly simple behaviors are not supported by more recent findings (Catania, 2000; Anjum et al., 2006), and additional studies of shrew brains and behavior will provide a more accurate understanding of this unique family of mammals. The American water shrew (*Sorex palustris*) typifies some of the dichotomies suggested above. Although weighing only 12-15 grams, this so-called "primitive" species is the smallest mammalian diver and can navigate and efficiently locate prey in both terrestrial and aquatic habitats (Calder, 1969). It is an aggressive predator that can quickly detect and subdue prey, including a range of invertebrates and fish. It forages primarily at night (Sorenson, 1962 and see Rychlik, 2005 for *Neomys*) and has been shown to localize and attack water movements that simulate escaping prey in only 50 milliseconds (Catania et al., 2008). It can also use its vibrissae to quickly discriminate stationary prey based on surface features alone (see also Anjum et al., 2006 for Etruscan shrew hunting behavior). Finally, it makes use of olfaction underwater by exhaling and re-inhaling air bubbles while foraging (Catania, 2006). This suite of behaviors makes water shrews of particular interest for examining brain organization.

In this study we investigated the chemoarchitecture of isocortex in the American water shrew. The present investigation stems from a fortuitous opportunity to examine flattened sections of juvenile water shrew isocortex (see materials and methods). In small mammals, juvenile cortex often reveals a particularly clear view of the architecture of layer 4, allowing sensory areas to be distinguished. This was the case for water shrews; when the tissue was processed for the metabolic enzyme cytochrome oxidase, cortical subdivisions were revealed that could be tentatively related to sensory representations based on previous electrophysiological experiments (Catania et al., 1999) supplemented with an additional microelectrode recording case in the present study. These findings allowed us to quantify the size of some cortical representations and these results are compared to selected cranial nerve counts to provide evidence for the dominant senses used by this species to explore its environment. In addition, derived features of shrew cortex are discussed in relationship to other insectivores and other mammals.

Materials and Methods

Adult water shrews (*Sorex palustris*) were collected with Sherman live traps in Potter and Cameron County, Pennsylvania under permit COL00087. Animals were housed in Plexiglass cages containing peat moss, sphagnum moss, soil, water bowl and were fed fish (*Pimephales promelas*), mealworms, crickets, wax worms, and canned cat food. One female water shrew gave birth to a litter of 3 young. To examine juvenile cortex, two young were removed from the nest, one at postnatal day 12 (weight 9.8

grams), and the other at postnatal day 13 (weight 10.3 grams). Each animal was given an overdose of sodium pentobarbital (at least 120 mg/kg) and perfused transcardially with phosphate buffer (pH 7.4) followed by 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS). The brain was removed and post-fixed in 4% PFA for at least 30 minutes. The cortical hemispheres were separated from underlying white matter and sub-cortical tissue. Additional adult water shrew specimens used in measurements of brain and body mass were kindly provided by Kevin Campbell of the University of Manitoba, Canada. Procedures met guidelines set by the National Institutes of Health, the Animal Welfare Act, and the Vanderbilt University Institutional Animal Care and Use Committee.

Cortical recording procedures

One adult male water shrew (11.8 grams) was anesthetized with a combination of urethane (15% weight/volume in distilled water) and ketamine (one-tenth diluted 100 mg/ml stock in saline), using an intraperitoneal injection of 0.05 ml of the urethane solution and an injection of 0.01 ml of ketamine solution given approximately 15 minutes later. Supplemental injections of ketamine at the same dosage were given to maintain a deep plane of anesthesia. The cortical surface contralateral to the recording hemisphere was exposed and the skull stabilized with dental acrylic to a metal post. The exposed cortex was covered with silicon fluid and photographed (Zeiss AxioCam HRC; Zeiss, Jena, Germany) using a Wild Photomakroskop M400 (Wild Heerbrugg, Gais, Switzerland). Each electrode penetration was marked on the photograph. Multiunit microelectrode recordings were made in the middle layers of cortex using low-impedance tungsten microelectrodes (1.0 MΩ at 1000 Hz). Neuronal responses were amplified and monitored on a speaker system as tactile, auditory, and visual stimuli were presented. Somatosensory stimuli consisted of small wooden probes and von Frey hairs touched to the skin surface or used to deflect whiskers. Auditory stimuli included clicks at various locations around the shrew. Visual stimuli included moving bars of light produced by an ophthalmoscope and obscuring light sources to produce moving shadows within the animal's visual field. Selected electrode penetrations were lesioned with a 10 µA current for 15 seconds while the electrode was withdrawn from the cortex at 50 µm/second.

Cortical preparations:

Fixed cortices from the adult and juvenile water shrews were flattened between glass slides and immersed in 30% sucrose in phosphate buffer for 12 hours. The flattened cortex was frozen, pia side down, on a flat ice block that had been trimmed with a microtome knife to be parallel to the knife edge. Sections were cut at 50-60 µm thickness and processed for the metabolic enzyme cytochrome oxidase (CO) according to Wong-Riley and Carrol (1984). Sections were photographed using a Zeiss AxioCam HRc digital camera (Zeiss, Jena, Germany) mounted onto a Zeiss Axioskop microscope using Zeiss Axiovision 4.5 software (Carl Zeiss Microimaging, Thornwood, NY, USA). Imported images were adjusted for brightness and contrast using Adobe Photoshop CS3 (Adobe Systems Incorporated, San Jose, CA, USA). Typically 3 cortical sections per hemisphere were optimal to be used to reconstruct the boundaries of sensory cortex using a light-projecting microscope (Bausch and Lomb, Rochester, NY, USA). Measurements from these drawings were made using ImageJ (National Institutes of Health, Bethesda, MD, USA).

Cranial nerve preparations:

For counts of myelinated fibers in cranial nerves II, V, and VIII (cochlear branch) tissue was collected from adults as it became available in the course of other studies. For cranial nerve II, both optic nerves from 3 adults (6 total) were processed and counted. For cranial nerve V, both trigeminal nerves from 2 adults were processed and counted (4 total). For cranial nerve VIII, one cochlear branch from each of two adults, and both from an additional adult (4 total) were processed and counted. Segments of cranial nerves were dissected following perfusions with 4% PFA and immersed in phosphate-buffered 2.5% glutaraldehyde solution for least 24 hours. Samples were post-fixed in osmium tetroxide, dehydrated in a graded ethanol series, transferred into propylene oxide, and embedded in EMBed 812 (EM Sciences, Hatfield, PA, USA). For cranial nerve II, ultrathin sections approximately 90 nm in thickness were cut with a diamond knife (Diatome US, Hatfield, PA, USA) on a Reichert Ultracut E ultramicrotome and mounted on size 75 mesh copper grids. Sections were stained with uranyl acetate followed by lead citrate for 10 minutes each and imaged with a Philips CM12 TEM (Philips Research, the Netherlands). Images were compiled in Photoshop CS3 to form montages of the nerve. For cranial nerves V and VIII, semi-thin serial sections (0.5-1 μm) were cut transversely on the ultramicrotome and stained with 1% toluidine blue. Tissue was examined at 100X under light microscopy (Zeiss Axioskop), and digital images were captured (Axiovision 4.5) and compiled in Adobe Photoshop CS3 into complete montages. Myelinated axons were manually counted.

Results

Figure 1A illustrates the diving behavior of a water shrew as it pursues prey. Water shrews are capable of rapidly detecting and orienting towards prey, using both somatosensory and olfactory cues (Catania, 2006; Catania et al., 2008). They have large hindlimbs fringed with stiff hairs (Fig. 1B) that aid in swimming and their fur is water resistant – trapping air that provides insulation (Calder, 1969) and facilitating buoyancy (as indicated by the reflective sheen in figure 1).

Although we primarily focused on the details of cortical subdivisions and cranial nerves, we also measured brain weight and body weight in 6 adult water shrews to provide data for potential studies of encephalization in different mammalian lineages. The average body weight was 11.5 grams (SD 2.2) and average brain weight was 0.26 grams (SD 0.05). These values are similar to the value reported for a single specimen by Stephan et al., (1991) at 14.6 grams body weight and 0.28 grams brain weight.

The gross morphology and size of the water shrew brain is illustrated in figure 2. The brain was lissencephalic with prominent olfactory bulbs and a relatively large isocortical surface. To investigate the details of sensory areas in the cortex, the hemispheres were flattened, sectioned tangentially, and processed to visualize the metabolic enzyme cytochrome oxidase (Figs. 3 and 4). For reasons that are not obvious, the best results for this procedure are generally obtained in juvenile mammals. This was the case for the water shrews, as figure 3 represents the most distinctive set of cortical subdivisions yet revealed for a shrew brain. The two most obvious chemoarchitectonic features were the primary somatosensory (S1) representation of the whiskers (visible as a

Figure 1. The American water shrew (*Sorex palustris*). **A.** At roughly 12 grams, this species is the smallest mammalian diver capable of rapidly locating and capturing small prey underwater. **B.** A fringe of stiff hairs on the fore and hind limbs provide additional surface area for paddling underwater.

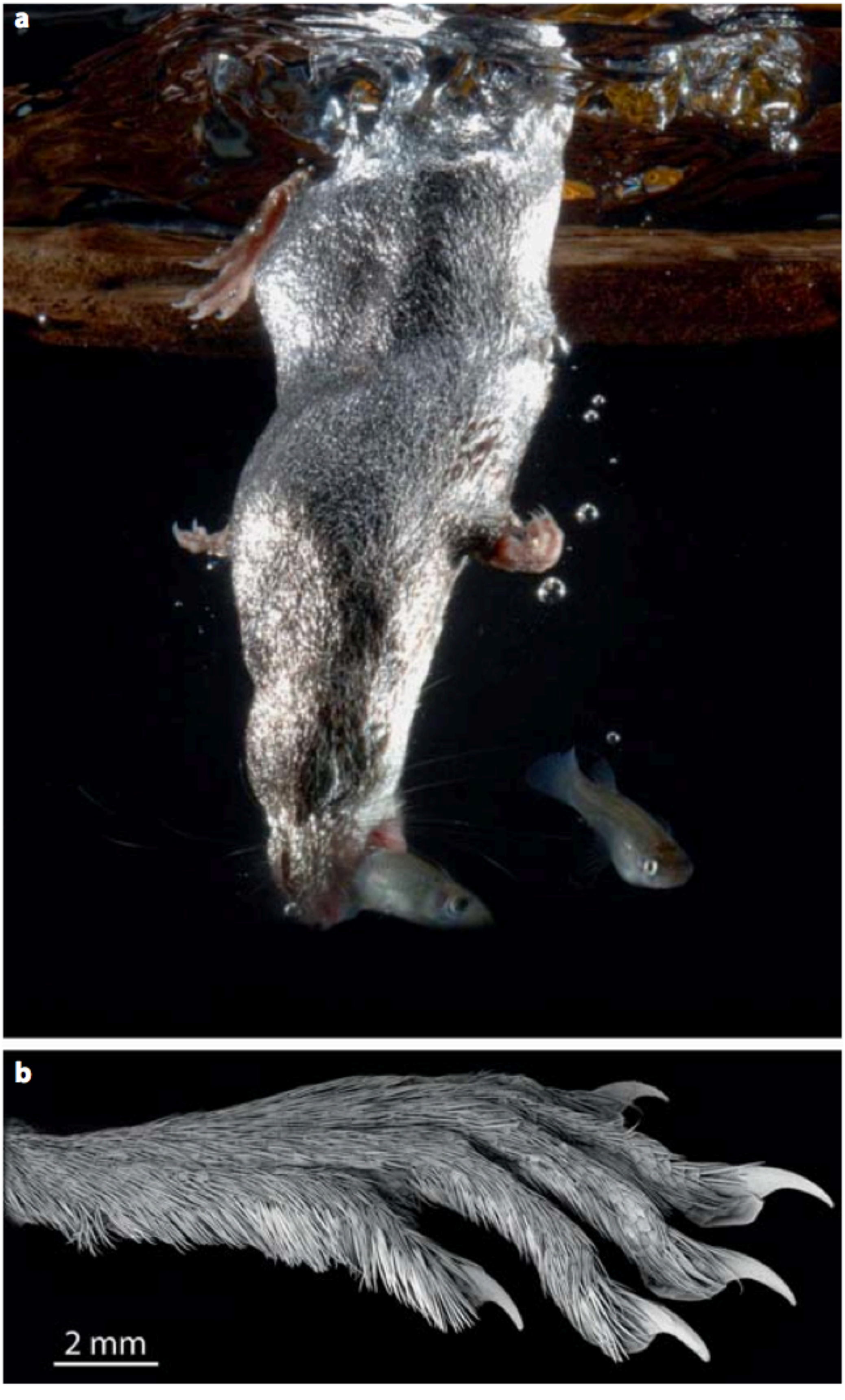

a
b
2 mm

Figure 2. Illustration of the Amercian water shrew brain. **A:** Side view. **B**: Dorsal view. BS, brainstem; OB, olfactory bulb.

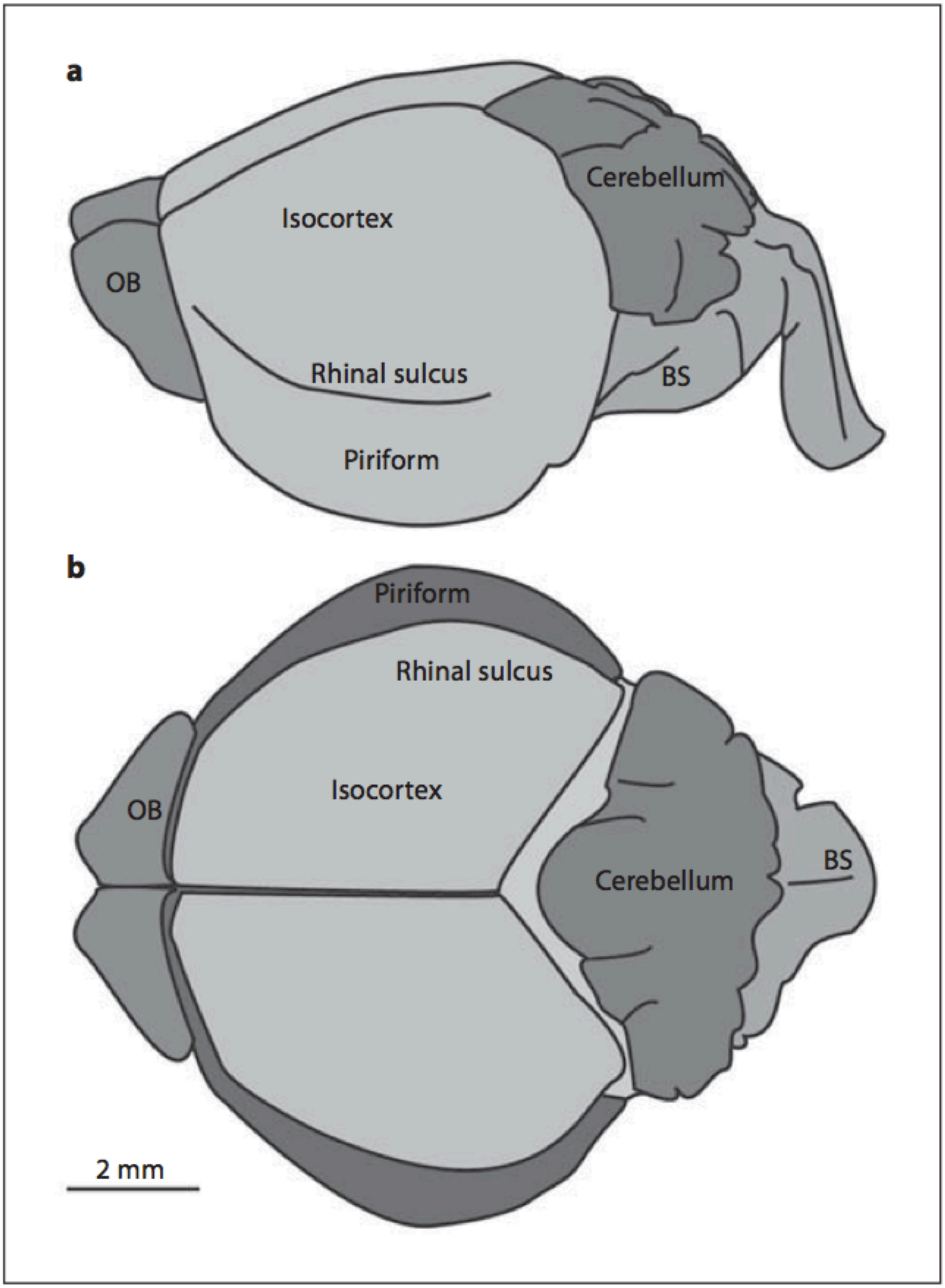

a
Cerebellum
Isocortex
OB
Rhinal sulcus
BS
Piriform
b
Piriform
Rhinal sulcus
Isocortex
OB
BS
Cerebellum
2 mm

Figure 3. Two consecutive sections from a flattened, juvenile American water shrew cortex processed for cytochrome oxidase. **A.** The large S1 vibrissae representation is obvious as a distinct dark wedge in middle cortex. A uniformly lighter region corresponds to the S2 vibrissae representation. The presumptive representation of oral structures is located at the rostral-lateral border of somatosensory cortex (see text). A portion of V1 (primary visual cortex) is visible in caudal cortex, but more obvious in its optimal plane of section in “B”. Arrowhead marks a light septum that typically separates the representation of oral structures (dark arrow) from the facial whiskers in shrews and moles (see text). **B.** An adjacent section reveals additional details. V1 is prominent caudally, closely bordering somatosensory areas. A small module (white arrow) marks the possible location of the lower jaw representation. A presumptive auditory area is located caudally and laterally. Rostral is left; medial is up. S1: primary somatosensory cortex; S2: secondary somatosensory cortex; V1: primary visual cortex; Aud; Auditory area; OB: olfactory bulb.

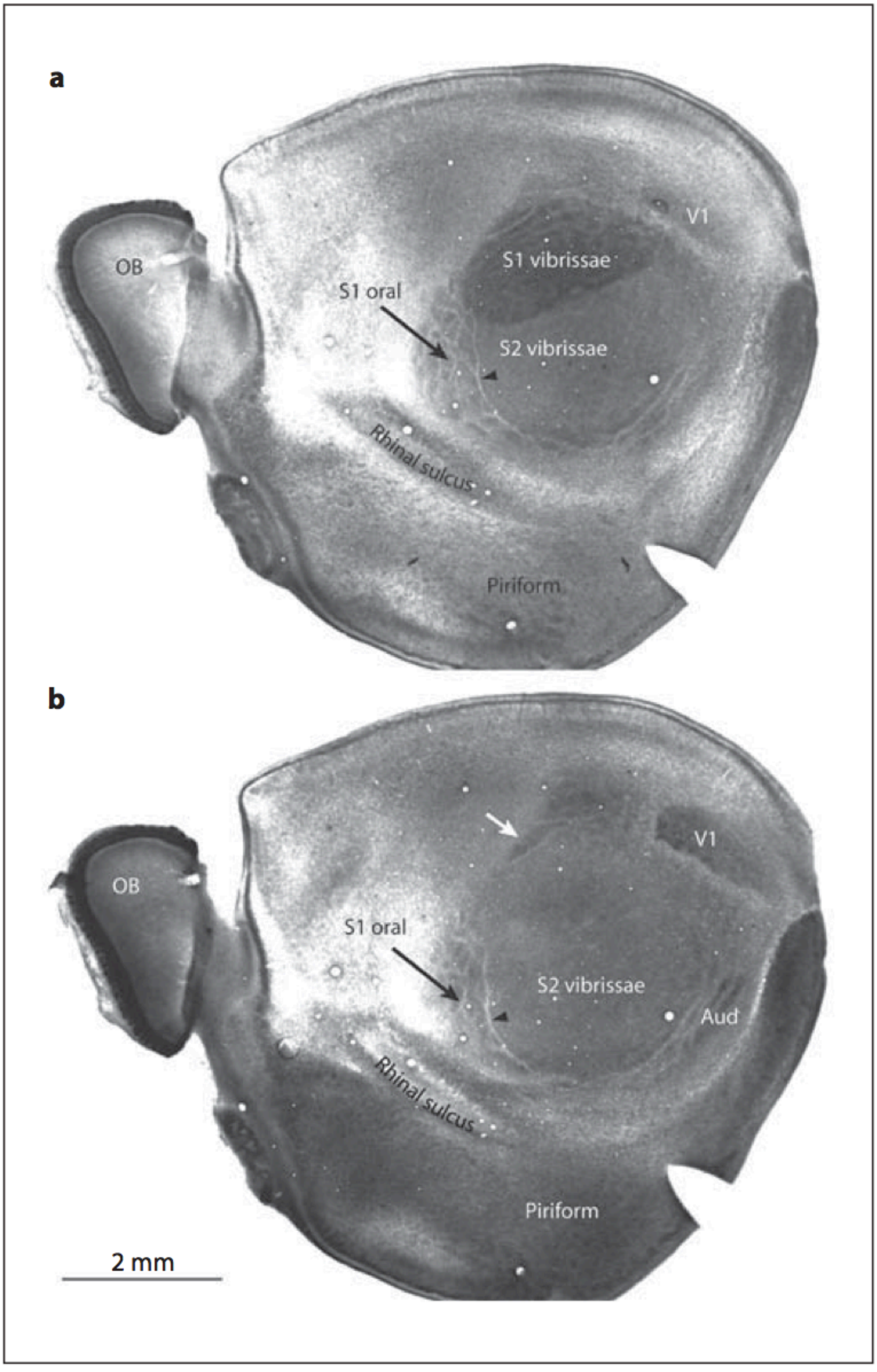
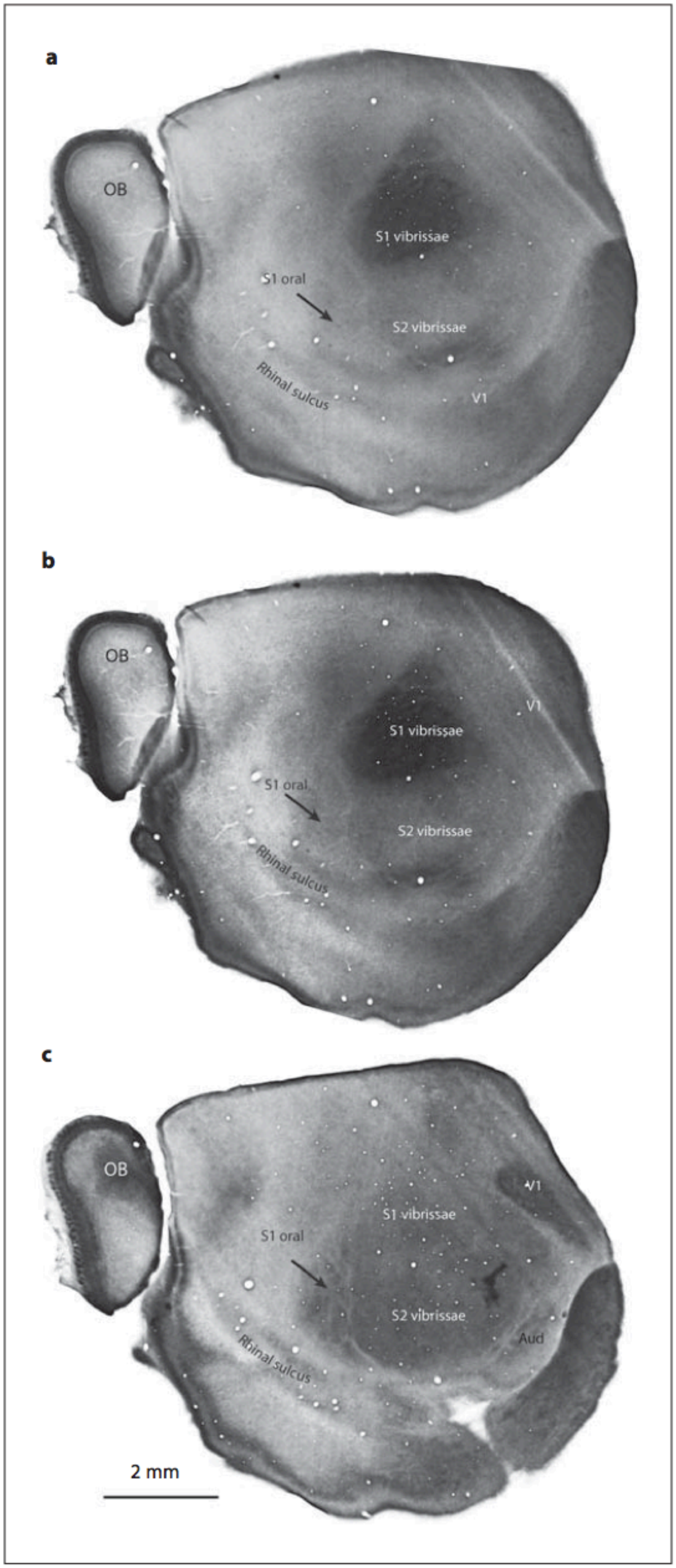

a
OB
V1
S1 vibrissae
S1 oral
S2 vibrissae
Rhinal sulcus
Piriform
b
OB
V1
S1 oral
S2 vibrissae
Aud
Rhinal sulcus
Piriform
2 mm

Figure 4. Three consecutive sections from the second flattened, juvenile American water shrew cortex processed for cytochrome oxidase. **A.** The prominent dark wedge corresponds to vibrissal representation in S1, bordering the S2 vibrissal representation. **B** and **C**. In the adjacent sections, V1 becomes more clearly visible, located directly caudal to S1 and S2 modules. Rostral is left; medial is up. S1: primary somatosensory cortex; S2: secondary somatosensory cortex; V1: primary visual cortex; OB: olfactory bulb.


a
OB
S1 vibrissae
S1 oral
S2 vibrissae
Rhinal sulcus
V1
b
OB
V1
S1 vibrissae
S1 oral
S2 vibrissae
Rhinal sulcus
c
OB
V1
S1 vibrissae
S1 oral
S2 vibrissae
Aud
Rhinal sulcus
2 mm

centrally located dark, heterogeneous wedge of tissue) and the primary visual cortex (V1) located more caudally (Fig. 3) in a location typical for V1 in other shrews, and similar to V1 in most other mammals with lissencephalic brains (Rosa and Krubitzer, 1999). The relative location of V1 in the American water shrew was considerably more medial than V1 previously identified in the masked shrew, *Sorex cinereus* (Catania et al., 1999). Primary visual cortex in an adult shrew in this study was located based on microelectrode recordings. A lesion made in the middle of the visually responsive zone corresponded to the center of a cytochrome oxidase-dark oval in caudal cortex (Fig. 5B). The same area was obvious in the juvenile cortex (Figs. 3 and 4). The identification of the S1 vibrissae region was also supported by microelectrode recordings from a single adult water shrew in the present study (Fig. 5) and from a single case in a previous investigation (Fig. 6).

A lighter staining region lateral to the S1 vibrissae representation generally contains a large S2 vibrissae representation that forms a mirror image of S1 in shrews. Microelectrode recordings in the water shrews (Figs. 5 and 6) and other shrew species (Catania et al., 1999) indicate that this region extends relatively far caudally and laterally, leaving only a small crescent shaped area of cortex responsive to auditory stimuli. We can identify this area as distinct from S1 because it contains a second representation of the vibrissae in shrews (Catania et al., 1999) and is chemoarchitectonically distinct from S1. Because this area represents the body as a mirror image of S1 in other shrews and moles (Catania, 2000), reflected along the snout, it has the characteristics of S2, the secondary somatosensory area found in a range of mammals (Huffman et al., 1999; Krubitzer et al., 1986; Johnson, 1990). Another distinctive feature of both shrew and mole (family Talpidae, the sister group to shrews) somatosensory cortex is a light

Figure 5. Results of a microelectrode recording experiment from single adult water shrew. **A.** Plot of electrode penetrations in different areas of the cortex. Red stars mark microlesions that allowed the alignment of mapping results with sections of cortex processed for cytochrome oxidase (CO). The green area indicates a CO-dense region corresponding to visual cortex. Rostral is left; medial is up. **B.** A portion of the flattened cortex processed for CO, showing the microlesions that designate penetration sites 6, 10 and 15, which was responsive to visual stimulation and its location within the darkly-stained V1 . Rostral is left; medial is up. **C.** Receptive fields on the body of the shrew that correspond to each electrode penetration in A.

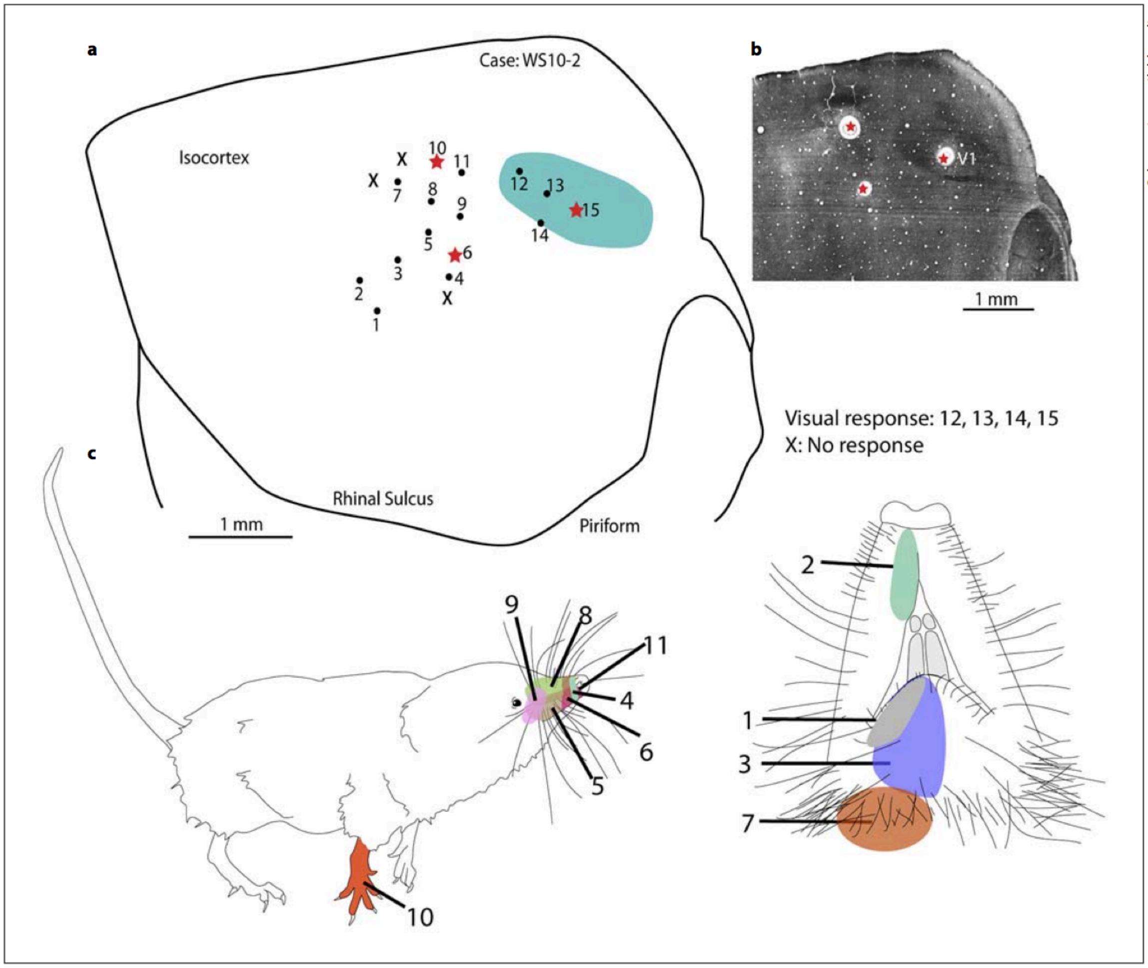
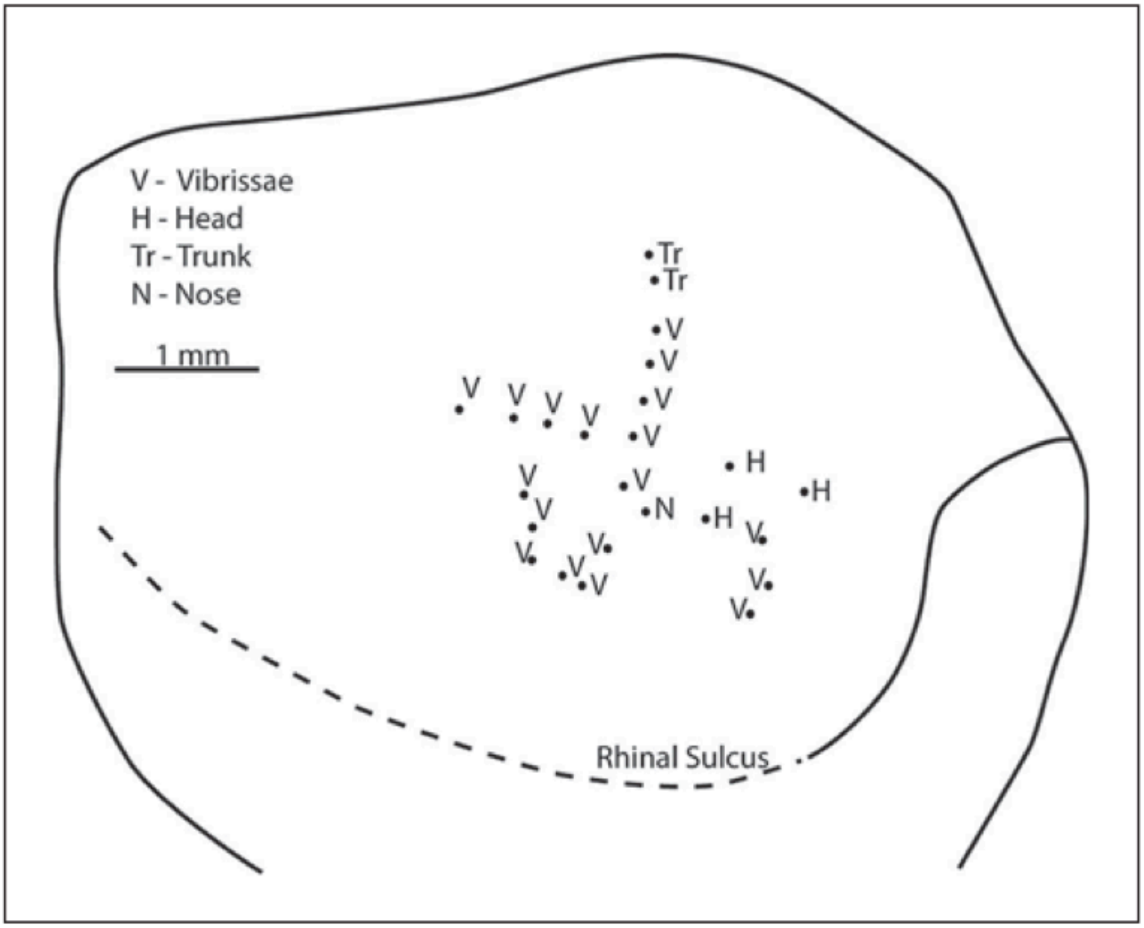

a
Case: WS10-2
Isocortex
X
X
10
11
7
8
9
5
6
3
4
2
X
1
12
13
15
14
Rhinal Sulcus
Piriform
1 mm
b
V1
1 mm
Visual response: 12, 13, 14, 15
X: No response
c
9
8
11
4
6
5
10
2
1
3
7

Figure 6. Summary of recording results from a single adult water shrew in a previous investigation (drawn from data in Catania et al., 1999).


V - Vibrissae
H - Head
Tr - Trunk
N - Nose
1 mm
Tr
Tr
V
V
V
V
V
V
V
V
V
V
V
V
V
V
V
V
V
N
H
H
H
Rhinal Sulcus

staining septum located rostrally and laterally in S1 (arrowheads, Fig. 3). This marks the border between the representation of vibrissae and oral structures in shrews and eastern American moles (*S. aquaticus*), or between the star representation and oral structures in the star-nosed mole (*C. cristata*) (Catania and Kaas, 1997; Catania, 2000). This septum was clearly evident in the cortical sections examined in the water shrew, and corresponds to a homologous area found in the short-tailed shrew (*Blarina brevicauda*) and the least shrew (*Cryptotus parva*) known to represent oral structures (see Catania et al., 1999). This rostro-lateral region in water shrews includes a series of small modules that likely represent different sensory surfaces in the oral cavity (dark arrow, Fig. 3)

More medially, a light arrowhead (Fig. 3B) indicates the possible representation of the lower jaw in S1. This small wedge is similar to the relative location and appearance of the wedge-shaped representation of the lower jaw visible in flattened mouse cortex processed for CO (Wallace, 1987). The forelimb representation would typically be located just medial to the lower jaw, and indeed a response from the forelimb was obtained and lesioned in the present investigation in the appropriated location from a single adult water shrew (Fig. 5, penetration 10). At the far caudo-lateral end of the isocortex, presumptive auditory cortex (A1) was represented by a small, dark crescent region bordered caudally and laterally by piriform cortex and bordered rostrally by the S2 whisker representation (Figs. 3 and 4). This interpretation is based on recordings and lesions from a homologous area in closely related shrews and moles (Catania et al., 1999).

Sensory cortex surface area comparisons

The sizes of cortical subdivisions were measured and compared to total neocortical area in the four cortical hemispheres from the two juvenile animals (Fig. 7). Serially-sectioned tissue was stained for cytochrome oxidase in order to draw and reconstruct the architectonic boundaries of these subdivisions. The measured areas included primary visual cortex (V1), somatosensory cortex (S1, S2), and total sensory cortex (S1, S2, V1 and auditory cortex). Somatosensory areas occupied the majority of sensory cortex (9.1 mm^2) and roughly a third of the total isocortical surface (29.9 mm^2). Within somatosensory cortex in both S1 and S2, large areas appeared to be devoted to representations of vibrissae. Bordering these areas, more heterogeneous regions separated by light staining septa indicate the likely representations of the trunk and limbs in rostromedial S1.

Cranial nerve size comparisons

In addition to examining cortical subdivisions, the number of myelinated afferents was counted for the optic, trigeminal, and cochlear nerves. Myelinated fibers could be readily distinguished at the light level for the trigeminal and cochlear nerves (Fig. 8A,B) whereas optic nerves required the use of transmission electron microscopy to collect images of adequate clarity (Fig. 8C). Among these three nerves, the trigeminal nerve, which transmits somatosensory information from the vibrissae, glabrous rhinarium, and other facial regions, was by far the largest, with a mean of 27,520 (SD 2890 axons from

Figure 7. Schematic illustration of different cortical subdivisions and their relative size in square millimeters for the 4 hemispheres from 2 juvenile water shrews. A-B. Juvenile water shrew 1 left cortex (A) and right (B) cortex. C-D. Juvenile water shrew 2 left (C) and right (B) cortex. B. Rostral is left; medial is up. S: somatosensory areas; A: auditory area; V1: primary visual cortex.

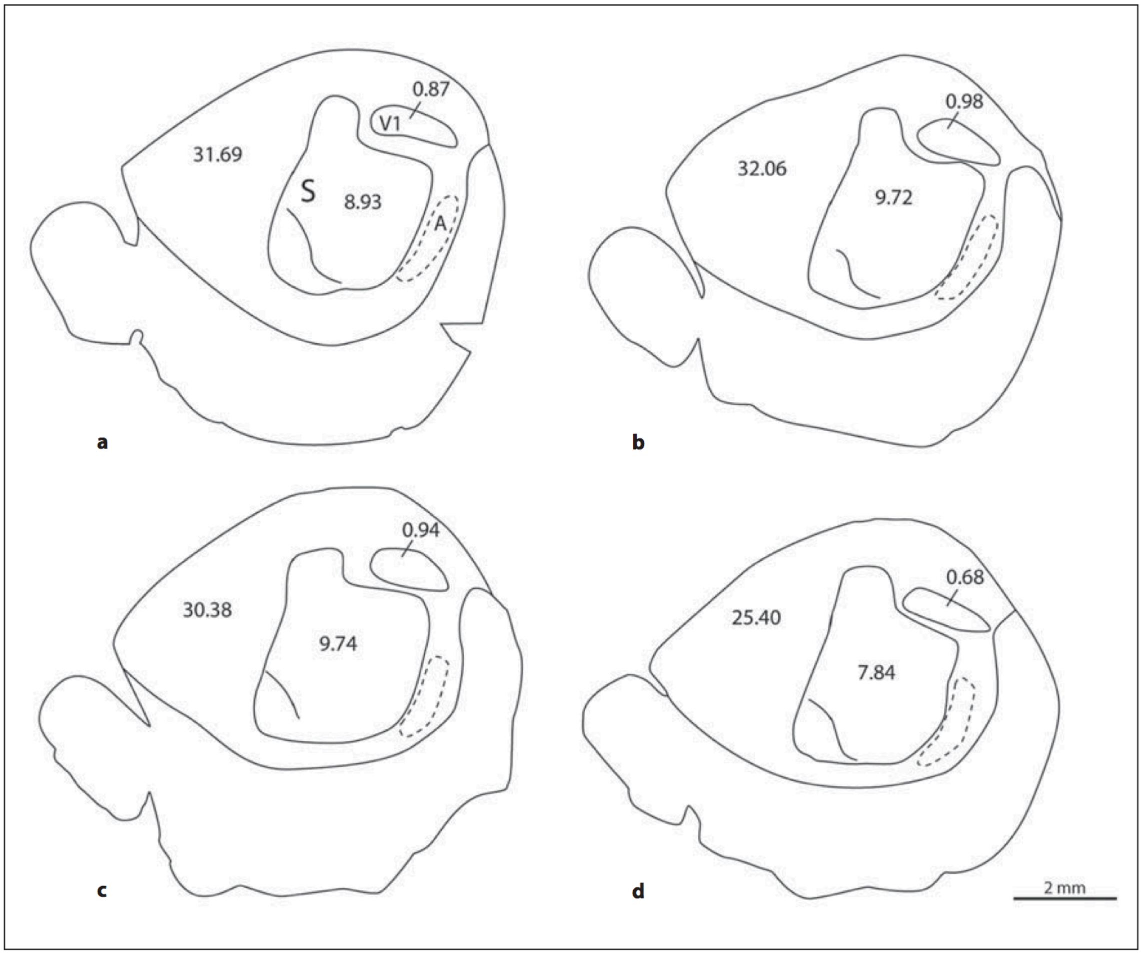
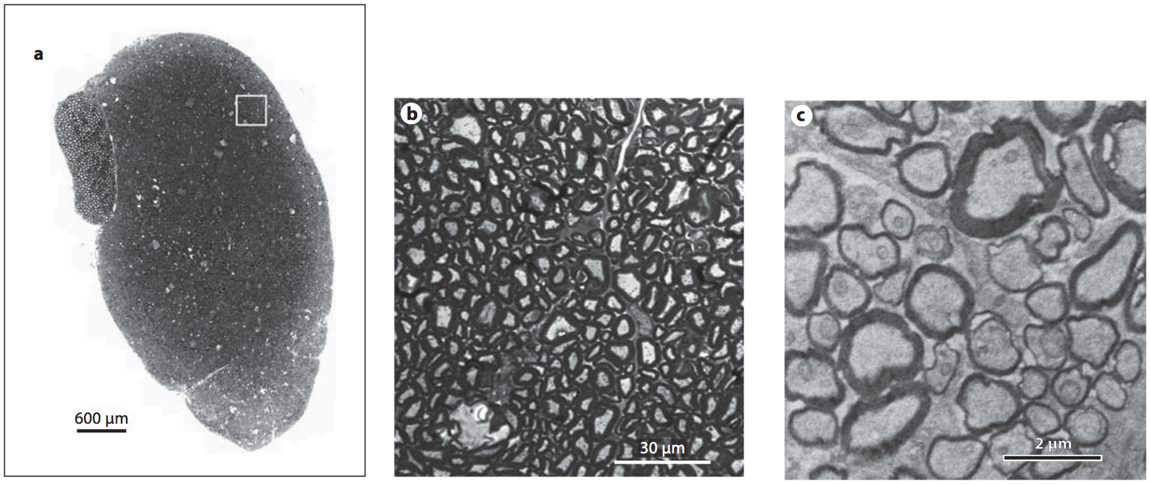

0.87
V1
31.69
S
8.93
A
a
0.98
32.06
9.72
b
0.94
30.38
9.74
c
0.68
25.40
7.84
d
2 mm

Figure 8. Cranial nerve preparations. A. Example of a complete montage of a transverse section of trigeminal nerve prepared in thick sections stained with toluidine blue and viewed under light microscopy. B: Higher magnification (from box in “A”) showing the distinctive myelinated fibers. C: Example of a single representative image from the montage taken with the transmission electron microscope and used to count myelinated fibers in the shrew’s optic nerve.


a
600 µm
b
30 µm
c
2 µm

the 7 counted samples. Both the optic and cochlear nerves were surprisingly small. The optic nerve contained a mean of 6,340 (SD 703) myelinated axons whereas the cochlear nerves contained approximately 7,040 (SD 687) myelinated axons. Thus together the cochlear and optic nerves contained less than half of the number of myelinated axons found in the trigeminal nerve.

Discussion

In this investigation we examined flattened cortex from juvenile water shrews to identify cortical subdivisions and compared these results to counts of several cranial nerves that supply the sensory information to the identified cortical areas. By examining the chemoarchitecture of flattened water shrew cortex and comparing these results to electrophysiological recordings and previous anatomical investigations of other shrew species (Catania et al., 1999) we were able to tentatively identify a number of cortical subdivisions. The results are consistent with behavioral observations and suggest that water shrews depend heavily on somatosensation and far less on vision and audition. Their isocortex was dominated by the large primary and secondary somatosensory areas (S1 and S2). The S1 vibrissae and mouth representations were clearly evident within the larger somatosensory region. A secondary representation of the vibrissae was identified based on its similar architectonic appearance to the S2 vibrissae in other shrew species, and based on microelectrode recordings (Figs. 5, 6 and Catania et al., 1999). Primary visual cortex was located in a position typical for a range of small mammals but was quite small. This is consistent with the size of the water shrew’s optic nerve (6,300 myelinated fibers) suggesting that vision is poorly developed. Because aquatic prey - such as larval insects, small fish, and larval amphibians - comprise a portion of the water shrew's diet (Hamilton, 1930) and these shrews often hunt at night (Sorenson, 1962) it is unlikely that vision is relied upon for successful prey capture. Conaway (1952) reported that water shrew vision seemed to be poorly developed and animals had difficulty locating non-moving minnows. Covering the water shrew's eyes had no appreciable effect in the animal's ability to rapidly locate hiding areas in terrestrial environments such as hollowed logs or tunnels (Sorenson, 1962). These earlier studies are consistent with our more recent investigation (Catania et al., 2008) showing that water shrews take the same amount of time to capture live fish under either infrared or full spectrum illumination indicating that eyesight is not required for this species to capture fast moving prey.

Despite superficially resembling water shrews, similarly sized laboratory mice have been shown to use visual cues to orient themselves in aquatic and terrestrial environments in a variety of experimental paradigms that measure visual acuity (Prusky and Douglas, 2003; Prusky et al., 2000). Common strains of lab mice (C57/BL6) have approximately 55,000 fibers within the optic nerve, although showing significant variation even among litters of the same strain (Jeon et al., 1998; Williams et al., 1996). Water shrews, like other shrew species (*C. parva, B. brevicauda, S. cinereus,* and *S. longirostris*), have a distinct V1 that is closely bordered by S1 and S2 (Figs. 3-4), leaving little room for additional visual areas (Catania, 2000). This is consistent with their relatively small-sized eyes, apparent lack of dependence on vision in hunting and navigation, and perhaps the constraints of a small isocortex, with V1 occupying roughly 3% of the 30 mm^2 total isocortical surface.

In contrast, behavioral evidence suggests the whiskers are particularly important in water shrews and other shrew species. For example, water shrews are able to rapidly discriminate cast prey items submerged in water under infrared lighting (Catania, 2008). Similarly, Etruscan shrews will accurately attack cast prey items on land (Anjum et al., 2006). In terms of afferents, the water shrew has a pronounced trigeminal nerve with approximately 28,000 fibers dedicated to relaying somatosensation from the facial regions. These measurements are similar to those from laboratory mice, which have about 26,000 fibers (Albers et al., 1996). Another small semi-aquatic mammal, the Australian water rat (*Hydromys chrysogaster*), has an impressive array of vibrissae and has been shown to have increased innervation of the follicle-sinus complex via the deep vibrissal nerve in comparison to terrestrial rodents (Dehnhardt et al., 1999). These results suggest that mystacial vibrissae can be effective in relaying hydrodynamic tactile information. At the level of the cortex, a distinctive CO-dense wedge-shaped area corresponded to the location of the whisker representation in primary somatosensory cortex (Fig. 3-4). Interestingly, this area did not contain cortical barrels. This provides a contrast to the condition in small-brained rodents, which typically have prominent barrels in somatosensory cortex, each delineating the representation of a single whisker (Woolsey and Van der Loos, 1970; Woolsey et al., 1975). It should be noted in this regard that the absence of barrels is not a characteristic of all insectivores, as the eastern American mole has barrels representing its smaller whiskers (Catania and Kaas, 1997).

In addition, star-nosed moles have a module representing each nasal appendage, although these appear as stripes and bands, rather than circular barrels (Catania et al., 1993).

Water shrews have small pinnae, as is typical of shrews generally (Churchfield, 1990). In laboratory settings, they have been recorded darting for cover in response to noises approximately 8 feet away (Sorenson, 1962). The cochlear component of the vestibulocochlear nerve has approximately 7,000 fibers, fewer than the 10,000 fibers reported in the laboratory mouse strains (Anniko and Arnesen, 1988). Although some shrews have been reported to produce ultrasound (Churchfield, 1990; Simeonovska-Nikolova, 2004; Siemers et al., 2009) American water shrews do not appear to emit such vocalizations above or below water (Catania et al., 2008). In comparison to the cortex of echolocating bats (Kössl and Vater, 1985; Suga et al., 1987; Vater et al., 1985) the chemoarchitectonically-defined auditory cortex of shrews is relatively small and its location and shape appear unique to shrews and moles (Catania, 2000).

The overall organization of water shrew isocortex is quite similar to that described in other shrews (Catania et al., 1999; Roth-Alpermann et al., 2010) and exhibits both conserved and derived traits when compared to other mammals. For example, the overall positions of V1, S1, and auditory cortex are similar among shrews and other small mammals with V1 located in caudal and somewhat medial cortex, S1 more rostrally located (with head and mouth represented more laterally and limbs and trunk represented more medially) and auditory cortex found caudo-laterally. However two unusual features stand out in shrew cortex. The first is a large S2 nearly equal in size to S1 (Catania et al., 1999; Roth-Alpermann et al., 2010). The second is a crescent-shaped auditory cortex at the caudal and lateral extreme of S2. Both of these unusual features are also found in the moles of the family Talpidae, but not in hedgehogs (Catania et al., 2000) or most other small mammals. These shared derived features of cortex provide support for the suggestion that Soricidae and Talpidae have a close taxonomic relationship.

The reasons for these unusual features are not entirely clear but some possibilities may be suggested. In the case of the small auditory cortex, the most obvious explanation is less reliance on audition in moles and shrews compared to other small mammal species. This may be the result of a past (and present) history that includes subterranean habitats where sound attenuates quickly. These conditions, in combination with an expansion of somatosensory cortex – particularly representations of vibrissae – may have compressed the small auditory cortex into its far caudal location. The reasons for the large S2 representation of sensory surfaces are less obvious. S2 in mammals is generally much smaller than S1 (Akers and Killackey, 1978; Huffman et al., 1999; Koralek et al., 1990; Remple et al., 2003; Wallace, 1987). One might speculate that S2 in shrews and moles has a role in parallel processing of touch that requires a strong thalamic input, much like area 3A in primates (Friedman and Jones, 1981; Krubitzer and Kaas, 1992). This could be readily explored with neuroanatomical tracers. It would also be useful to examine single unit responses from S1 and S2 in shrews to determine possible differential representation of mechanoreceptor classes in the skin and whiskers.

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CHAPTER III

Function of the appendages in tentacled snakes (*Erpeton tentaculatus*)

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Abstract

We investigated the function of the tentacles in aquatic, piscivorous tentacled snakes (Erpeton tentaculatus) by examining anatomy, peripheral innervation, and the response properties of primary afferents. We also investigated visual and somatosensory responses in the optic tectum and documented predatory strikes to visual stimuli and under infrared illumination. Our results show the tentacles are sensitive mechanoreceptors that respond to water movements. They are innervated by rami of the maxillary and ophthalmic branches of the trigeminal nerve and contain a dense array of fine terminal neurites that cross the interior of the tentacle orthogonal to its long axis. The optic tectum contained a retinotopic map of contralateral receptive fields with superior fields represented dorsally in the tectum, inferior fields represented laterally, nasal fields represented rostrally, and temporal fields represented caudally. Large somatosensory receptive fields were identified in deeper layers of the tectum and were in approximate

register with overlying visual fields. Tentacled snakes struck accurately at a simulated digital fish, indicating that visual cues are sufficient to guide strikes, but they also captured fish under infrared illumination, suggesting water movements alone could be used to localize prey. We conclude the tentacles are mechanosensors that are used to detect fish position based on water movements and that visual and mechanosensory cues may be integrated in the tectum to enhance localization when visual cues are reduced.

Introduction

Aquatic tentacled snakes (Erpeton tentaculatus Lacépède 1800) have a distinctive and unique pair of scaled facial appendages that project from the rostral margins of the head (Fig. 1). The function of the tentacles has been the source of speculation for over a century and they have variably been considered lures, aids to camouflage, ornaments or mechanoreceptors (Gunther, 1864; Smith, 1943; Shaw, 1965; Bellairs, 1970; Hahn, 1973; Winokur, 1977). Because tentacled snakes are fully aquatic and feed almost exclusively on fish (Murphy, 2007), appendages that detect water movements could potentially provide an important aid to localizing prey. This is particularly true in turbid water or at night when visual cues are poor, and this possibility has been suggested by a number of investigators (Morice, 1875; Winokur, 1977; Smith et al., 2002; Murphy, 2007). The hunting strategy of these snakes is well-suited to the detection of water movements generated by fish. They adopt a cryptic J-shaped posture and usually wait motionless to strike until fish have entered the concave region between their head and body (Smith et al., 2002; Murphy, 2007; Catania, 2009). Investigations of their striking behavior have revealed specializations for acceleration of the head in an aquatic medium (Smith et al., 2002) and a mechanism for startling fish toward the jaws using a pre-strike feint with the body (Catania, 2009). The latter behavior includes a predictive strike for some fish orientations, during which the snake aims for the future location of the escaping fish's head. This strategy is a testament to the snake's long evolutionary history of predation on fish. In light of these exceptional behavioral specializations, the unique facial appendages seem all the more intriguing. Yet there have been few studies of their anatomy and no investigation of their function from a behavioral or neurophysiological approach.

The first anatomical study of the tentacles reported a complete lack of innervation, suggesting no sensory role for the appendages (Hahn, 1973). However, a subsequent investigation of the tentacles by Winokur (Winokur, 1977), using Winkelmann's silver stain, reported a substantial innervation, supporting a potential sensory role. Our goal in this study was to determine the function of the tentacles by examining behavior, innervation of the tentacles, response properties of trigeminal neurons, and the mapping of visual and somatosensory responses in the optic tectum. Our results suggest the tenctacles are used to detect fish-generated water movements and suggest that mechanosenory and visual cues are integrated in the tectum.

Materials and Methods

Nine tentacled snakes were used in this study. They were housed in aquaria containing at least 30 cm of water, gravel and plastic plants, pH between 6.5 and 7, and fed Fathead minnows (Pimephales promelas) and goldfish (Carassius auratus). Water temperature was maintained between 24 and 28°C. All procedures conformed to National Institutes of Health standards concerning the use and welfare of experimental animals and were approved by the Vanderbilt University Animal Care and Use Committee.

Scanning electron microscopy

To examine tissue under the scanning electron microscope, animals were killed with sodium pentobarbital (120 mg kg–1) and perfused through the heart with 4% paraformaldehyde (PFA) and tissue was then immersion fixed for 24–48 h. Tissue was rinsed in phosphate buffered saline (PBS), dehydrated in ethanol, critical point dried in an E3000 drier (Quorum Technologies, Guelph, ON, Canada) and coated with gold in a Cressington 108 sputter coater (Cressington Scientific Instruments Ltd, Watford, UK). Specimens were viewed in a Tescan Vega II SEM (Tescan USA, Cranberry Twp, PA, USA).

DiI and confocal microscopy

Tentacles were removed post-mortem and stored in fixative (PFA) for at least 48 h. A sharpened wooden probe was then used to apply small crystals of DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; Molecular Probes Invitrogen, Carlsbad, CA, USA) to the cut, proximal end of the tentacle containing the exposed nerve. The tentacle was embedded in 2% agarose, immersed in 4% PFA, and stored in darkness for at least 4 weeks. The tentacle was then hemisected, placed on a slide with a drop of PBS, and coverslipped using tackiwax as a spacer. Images were collected on an upright LSM510 confocal microscope (Zeiss, Thornwood, NY, USA).

Sudan Black B

To identify the course of peripheral rami for the trigeminal nerves (Fig. 2C) the brain was removed from whole or hemisected heads, and the material was processed as outlined in Filipski and Wilson (Filipski and Wilson, 1984). Specimens were fixed in 10% formalin for 1 week, washed in tap water for 12 h, and then cleared in 10% hydrogen peroxide for 2–3 days, followed by a deionized water wash for 3 h. They were then macerated in a trypsin solution, transferred to 0.5% KOH for 10 min, 70% EtOH for 15 min, and then moved to a Sudan Black B solution (0.5 g Sudan Black B, Sigma Chemical Co, St Louis, MO, USA, in 100 ml of 70% EtOH) for 25 min. They were destained in 70% alcohol, transferred to 0.5% KOH for 12 h, and then dehydrated in glycerin containing 0.5% KOH.

Trigeminal and optic tectum responses

Snakes were anesthetized with a combination of urethane (0.4 g kg–1) and ketamine (100 mg kg–1) followed by supplements as needed. Once a surgical plane of anesthesia was reached the snake was intubated, manually respirated, and an incision was made to expose the skull. The head was secured to a post with two small screws and dental acrylic. A fine drill was used to expose either the optic tectum or the trigeminal ganglion. For recordings from the tectum, the tectum was photographed to mark electrode penetrations relative to surface features and blood vessels. Recordings were made in the tectum or trigeminal ganglion with low impedance tungsten microelectrodes (1.0–1.5 MΩ at 1000 Hz) using a Bak headstage and preamplifier (BAK Electronics, Inc., MT Airy, MD, USA) routed to a Neurolog amplifier and filters (Digitimer, Welwyn Garden City, Herts, UK). Recordings were monitored with a speaker and viewed on an oscilloscope. Single unit waveforms were sampled and stored at 100,000 samples s–1 using a Powerlab 4/30 attached to a Macintosh G4 laptop using Labchart 7.0 software

(ADInstruments, Colorado Springs, CO, USA). The Chubbuck stimulator (Chubbuck, 1966) was driven by a digital sine wave generator and a Master 8 digital stimulator (A.M.P.I. Jerusalem, Israel). Visual receptive fields were mapped by projecting small moving bars and circles of light, using an ophthalmoscope, onto the far side of a translucent, 45 cm diameter hemisphere placed flush with the snake's head and centered on the eye. The small size of the eye precluded identification of an optic disk or retinal streak. For somatosensory receptive fields, the skin was stimulated with a wooden probe or von Frey hair. After recordings, snakes were killed and perfused as described above. For tectal recordings, the tectum was removed, photographed, flattened on a freezing microtome, sectioned tangentially, and processed for cytochrome oxidase as described previously (Crish et al., 2003).

Behavior

Behavior trials were filmed with a MotionPro HS-3 camera (Redlake, IDT, Tallahasee, FL, USA) and video was transferred to a MacPro laptop using MotionProX software (Integrated Design Tools, www.idtpiv.com). The simulated digital fish was created in Adobe Illustrator (CS3) and moved by creating a Quicktime (Apple, Cupertino, CA, USA) movie that translated the image across a horizontally positioned Apple Cinema display – see supplementary material Movie 1. Infrared trials were filmed using two IR-Flood Ultra-Covert 940 nm illuminators (Night Vision Experts, Buffalo, NY, USA).

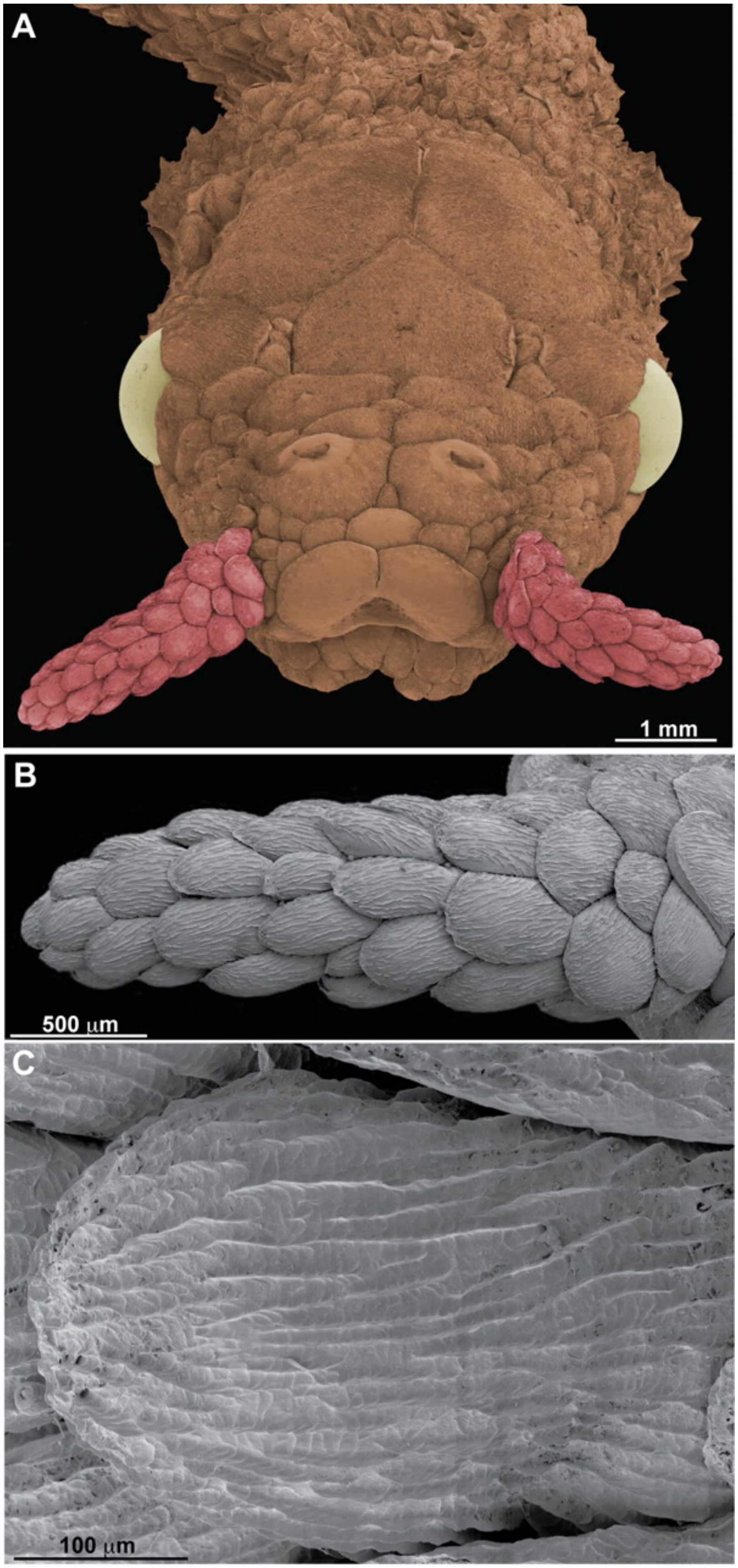
Results

Because the tentacles of Erpeton are a biological novelty of unknown function, we have examined a number of facets of tentacled snake sensory biology to provide evidence for their potential function and importance. The results start with a description of the peripheral anatomy, progress to an account of the trigeminal and tectal responses, and finally some behavioral observations are described with reference to Movie 1 in the supplementary material.

Tentacle structure and innervation

Fig. 1A shows the head and tentacles projecting from the face under the scanning electron microscope. The tentacles were covered with scales in all regions including the tips, and higher magnification (Fig. 1B,C) did not reveal scale sensillae (Povel and van der Kooij, 1997) or ampullary type organs – i.e. electroreceptors (Fritzsch and Wahnschaffe, 1983). Similarly, no evidence of such end organs in the epidermis was found in serial plastic sections stained with Toluidine blue or paraffin embedded sections processed for hemtoxylin and eosin or Masson's trichrome (not illustrated). As reported by Winokur (Winokur, 1977), the interior of the tentacle was composed largely of collagen fibers interspersed with smooth muscle and contained a number of blood sinuses and vessels. In a 25 cm long newborn snake, the tentacles extended approximately 4 mm from the 1.4 cm length head. In a 66 cm long adult, the tentacles were 6 mm in length extending from the 2.7 cm head. When the snake was waiting to strike, the tentacles projected from the face, usually at a roughly 45 deg. angle from the midline. They were highly flexible and folded to the side of the snake's head as strikes were initiated (see

Fig. 1. The tentacled snake head and tentacles viewed under the scanning electron microscope. **A.** The colorized head showing the eyes, dorsally located and closable nostrils, and paired tentacles. **B.** A single tentacle at high magnification showing scales covering all surfaces. **C.** A single scale typical of the tentacle. The scales are small, but similar to body scales and do not exhibit pits, ciliated hair cells, ampullary organs or projections.

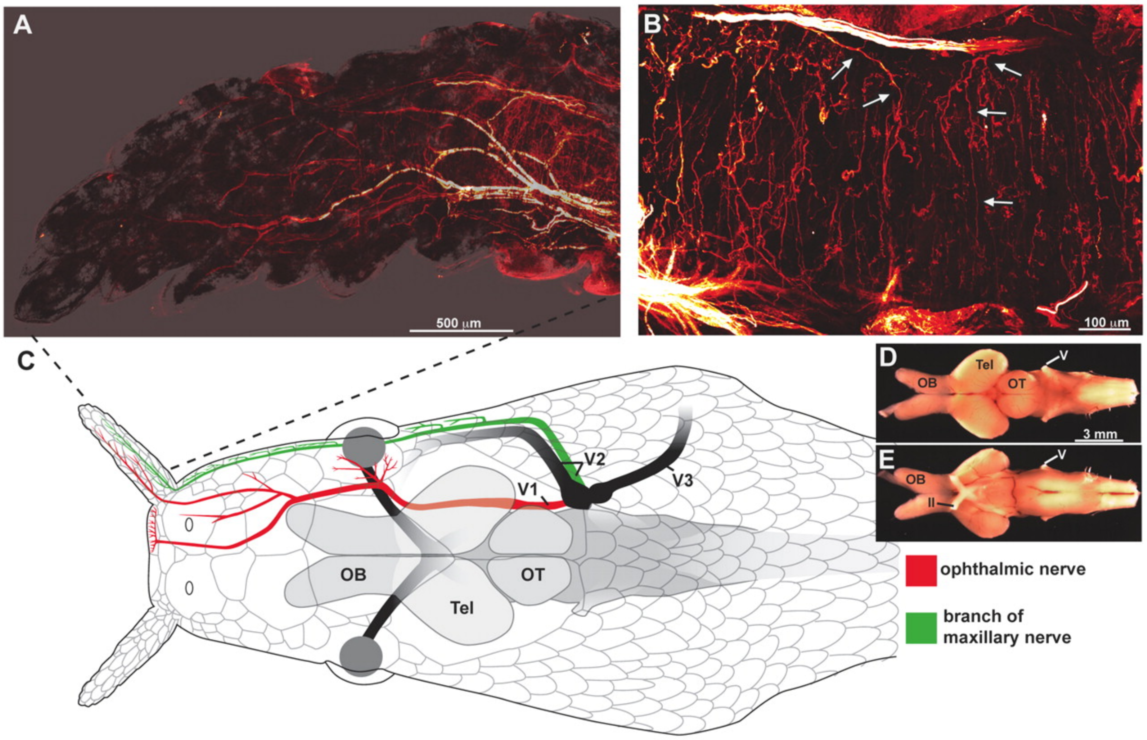

A
1 mm
B
500 μm
C
100 μm

Catania, 2009). Sections of the tentacles revealed several nerve trunks containing myelinated fibers but did not provide details of fiber distributions within the tentacles.

Application of the lipophilic neuronal tracer DiI to the proximal end of PFA fixed tentacles revealed details of their innervation. Fig. 2A is a composite of a transmitted light image and a confocal image of the transported florescent DiI in a tentacle that was hemisected through its long axis. The larger nerve trunks for the hemi-tentacle are visible in relation to the epidermis and scales. The individual scales of the tentacle were poorly innervated and relatively few fibers closely approached the keratinized surface. In contrast, a very dense network of fine fibers traversed the center of the tentacle in a direction almost uniformly orthogonal to its long axis. The extent of this fine network of fibers is obvious at higher magnification with a shallower focal plane (Fig. 2B) where many fibers are seen traversing the center of the tentacle, derived from the larger fascicles at the margins (arrows).

To determine the source of the tentacle's innervation, whole fixed heads were cleared and stained with Sudan Black B (Filipski and Wilson, 1984). The procedure revealed the cranial nerves and their rami in detail and allowed the nerves within the tentacle to be traced back to their origins. The results are shown in schematic form in Fig. 2C. As is the case for the pit organs in crotalines (Molenaar, 1992) different trigeminal nerves supplied the tentacle. Both the maxillary nerve and the ophthalmic nerve provided roughly equal densities of innervation. The same branch of the maxillary nerve that supplied the tentacle provided a dense innervation to the labial portion of the upper jawand the nerve became progressively smaller along its route as repeated portions

Fig. 2. Innervation of the tentacles by the trigeminal nerve. **A.** Composite image of the tentacle under transmitted light and confocal fluorescence of DiI labeled nerve fibers showing its relatively dense innervation. **B.** Higher magnification reveals a dense network of fine fibers that cross the middle of the tentacle orthogonal to the long axis. The fibers (arrows) are derived from larger branches at the margins. **C.** A schematic diagram of the head, brain and selected cranial nerves. Two different subdivisions of the trigeminal nerve (the ophthalmic and a branch of the maxillary) supply roughly equal densities of innervation to the tentacle. Nerves were traced from Sudan Black B preparations (see Materials and methods). V1–3, trigeminal nerve; OB, olfactory bulb; Tel, telencephalon; OT, optic tectum. **D.** Dorsal view of the brain showing the olfactory bulb, telencephalon, optic tectum and root of the trigeminal nerve. (E) Ventral view of the brain showing substantial optic nerve (II).


A
500 μm
B
100 μm
C
V2
V1
V3
OB
OT
Tel
D
OB
Tel
OT
V
3 mm
E
OB
II
V
🟥 ophthalmic nerve
🟩 branch of maxillary nerve

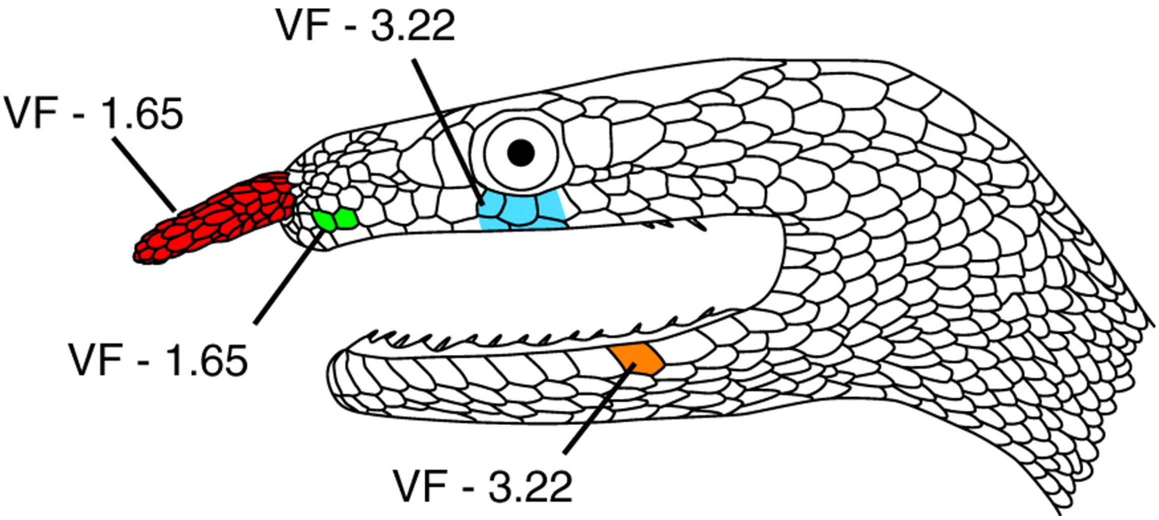
branched to supply the labial area (Fig. 2C, green). Only a relatively small fraction of this maxillary branch ultimately supplied the tentacle. The ophthalmic nerve also branched extensively and more diversely along its course (Fig. 2C, red) until only a comparatively small proportion of the fibers supplied the tentacle. In addition to innervating the tentacle distally, the ophthalmic nerve densely innervated the labial portion of the distal face medial and adjacent to the tentacle. Although the tentacle was well-innervated, it should be emphasized that the entire labial region of the upper jaw was densely innervated.

Afferent responses recorded from the trigeminal ganglion

We recorded extracellular activity from afferents at the level of the trigeminal ganglia in five cases to provide direct evidence of how the receptors on the face and tentacle responded. Receptive fields were generally small (Fig. 3) and it was immediately obvious that the tentacles were very sensitive to tactile stimulation, as was the entire labial region of the upper jaw and midline face. When the electrode was in the appropriate location slight deflection of the tentacle resulted in a strong multiunit response and single units were readily isolated for more detailed analysis. von Frey hairs were used to establish sensitivity thresholds for some units, and afferents innervating the tentacle responded to pressure applied with a 1.65 filament corresponding to a force of 0.008 g. Lower thresholds could not be determined as this was the smallest calibrated filament available.

Single unit responses to stimulation with a Chubbuck mechanosensory stimulator (Chubbuck, 1966) were recorded to investigate the reaction to direct contact of the stimulator probe to the skin. The stimulator provided a precise measure of probe motion

Fig. 3. Selected receptive fields of single trigeminal afferents recorded from the trigeminal ganglion. The numbers represent the finest von Frey (VF) hairs for which responses were obtained.

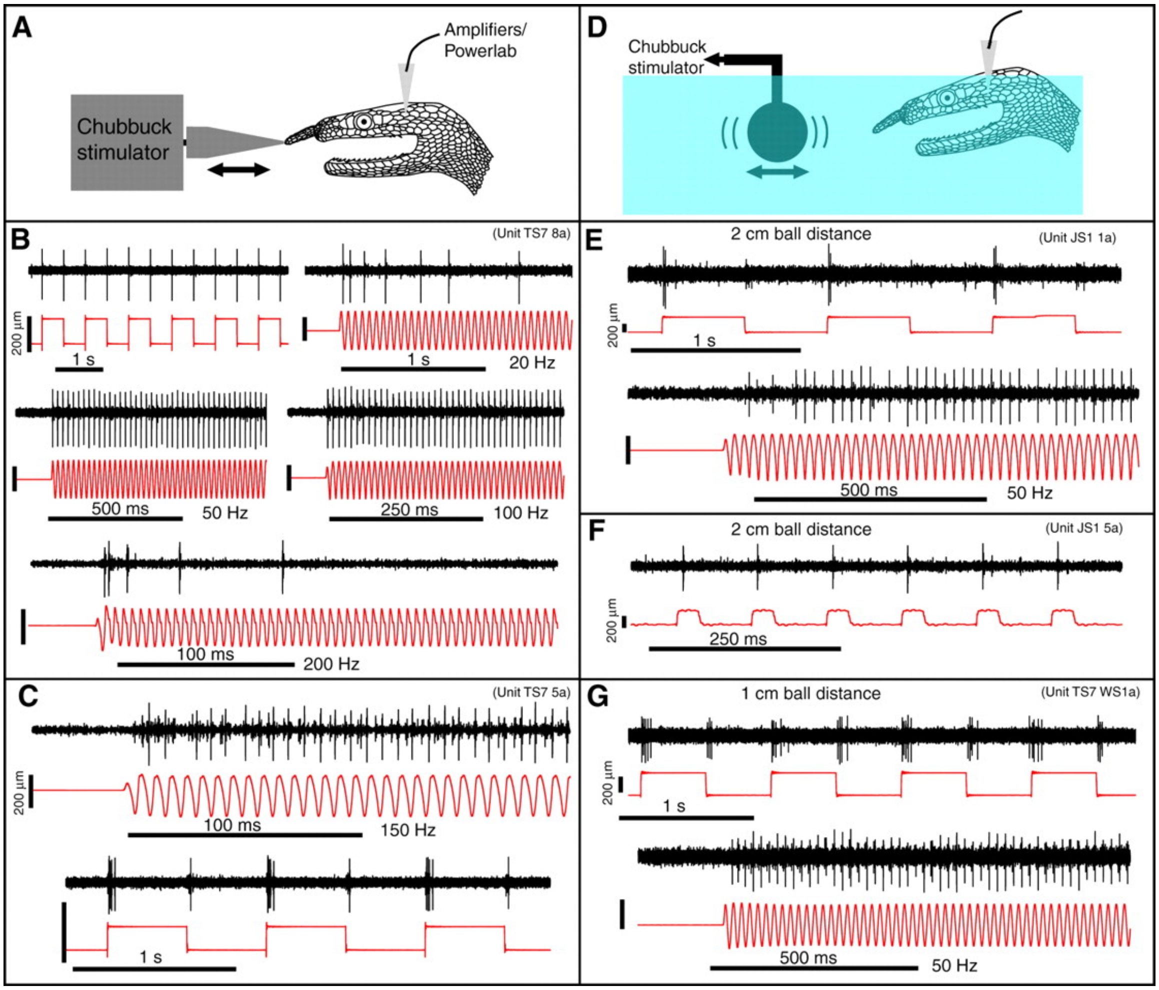

VF - 3.22
VF - 1.65
VF - 1.65
VF - 3.22

that was recorded in conjunction with neuronal responses. Examples of responses from units with receptive fields on the tentacle are illustrated in Fig. 4A–C. Responses were obtained to the onset and offset of tentacle deflection and were generally phase locked to stimulus movement for sinusoidal vibrations in the appropriate range. Most units were rapidly adapting and responded with one or a few spikes to stimulus onset and a single spike to stimulus offset (Fig. 4B,C). Rapidly adapting responses were maximal with a phase locked one-to-one response per cycle to vibrations in the 50–150 Hz range and were poor to 20 and 200 Hz stimuli.

To determine whether the tentacles responded to water movements, the snake's head was submerged in water and a 1.4 cm diameter sphere was attached to the stimulator (Fig. 4D) and submerged at a distance of 1 or 2 cm from the head.. Units were isolated with receptive fields on the tentacle, and the sphere was driven by the stimulator with square waves and sinusoidal vibrations as described above. The tentacle afferents readily responded to water movements generated at distances of 1–2 cm with movements of the sphere in the 300–400 μm range (Fig. 4E–G). In some cases, responses were obtained from neurons with receptive fields on the labial region of the face (not illustrated).

In the course of these experiments, we tested for responses to weak electric fields in the water using currents of 50–150 μA between two metal electrodes. Constant current was used to avoid transients generated by square wave stimuli; however, the electrodes were moved around the head in a range of configurations with both cathode and anode sequentially placed closer to the animal. No single or multiunit activity was observed or recorded for any stimulus strength or configuration. A 9 V battery was also placed in the

Fig. 4. Results of recordings from the trigeminal ganglia for direct contact of the tentacle with a Chubbuck mechanosensory stimulator (A–C) and for water movements generated by a submerged 1.4 cm diameter sphere driven by the stimulator (D–G). **A.** Schematic illustration of the model for trigeminal recordings from direct mechanosensory stimulation. The Chubbuck stimulator is a dedicated mechanosensory stimulus delivery unit designed to provide precise feedback of the probe location that can be recorded on a separate channel (Chubbuck, 1966). The red trace below each unit in B and C indicates probe movement in relation to action potentials (above). **B.** Recording of a single trigeminal unit (black) responding to excursion of the probe tip (red) driven by a square wave and a series of vibratory stimuli driven by sinusoidal voltages. Most units responded maximally to vibration in the 50–150 Hz range with spikes phase locked to the stimulus. **C.** A second unit responding to a square wave driving stimulus and phase locked to a 150 Hz vibration. **D.** Schematic illustration of the model for trigeminal recordings for water movements. **E–G** illustrate three different units responding to movement of the sphere at a distance of 2 cm **(E,F)** or 1 cm **(G).** Conventions as in B,C.


A
Amplifiers/
Powerlab
Chubbuck
stimulator
D
Chubbuck
stimulator
B
(Unit TS7 8a)
200 µm
1 s
1 s
20 Hz
500 ms
50 Hz
250 ms
100 Hz
100 ms
200 Hz
C
(Unit TS7 5a)
200 µm
100 ms
150 Hz
1 s
E
2 cm ball distance
(Unit JS1 1a)
200 µm
1 s
500 ms
50 Hz
F
2 cm ball distance
(Unit JS1 5a)
200 µm
250 ms
G
1 cm ball distance
(Unit TS7 WS1a)
200 µm
1 s
500 ms
50 Hz

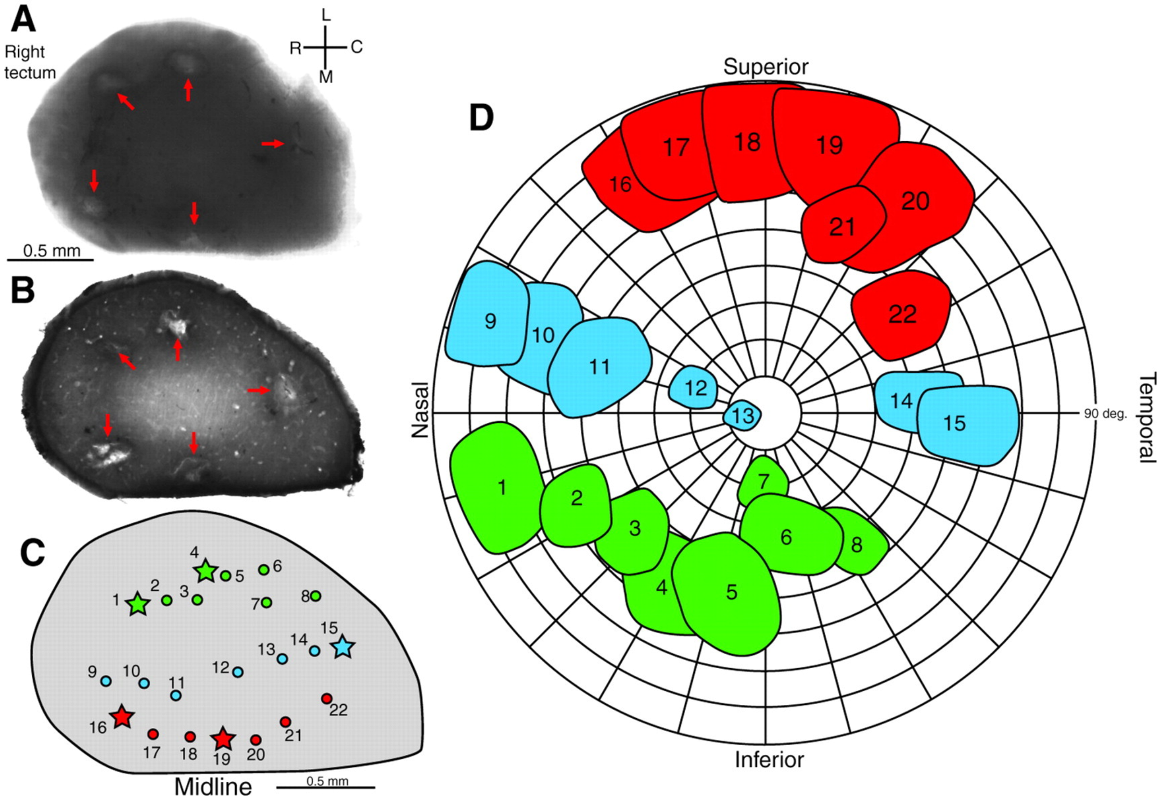
water and moved around the head. Although the currents generated by this stimulus are far above the appropriate range for electroreception, we reasoned that any potential electroreceptors would nevertheless be stimulated, much as a bright flash would stimulate photoreceptors [see also Scheich et al. for responses to battery in platypus (Scheich et al., 1986)]. No single or multiunit activity was observed.

Recordings from the optic tectum

The tectum was well-developed and obvious in brain dissections (Fig. 2D) and our initial goal in recordings was to assess responsiveness to visual stimuli and to determine the topography of retinal projections. To facilitate correlation of responses to the medio-lateral and rostro-caudal dimensions of the tectum, we made lesions at selected penetration sites during our recording sessions and later processed the tissue by dissecting the tectum from the brain (after fixation), identifying the lesioned sites (Fig. 5A), and then gently flattening the tectum on a freezing microtome. The tectum was then sectioned in the tangential plane so that lesions could be visualized relative to the entire areal extent of the tectum (Fig. 5B). The lesions were then aligned, in schematic form, with the locations of all electrode penetrations from a photograph of the tectal surface on which electrode penetration had been marked (Fig. 5C).

As might be expected, the tectum was very responsive to visual stimuli presented on a translucent hemisphere used to map receptive fields. Strong visual responses were obtained from superficial penetrations down to a depth of several hundred micrometers. Most visual receptive fields were mapped at a depth of 50–100 μm. Because our first goal was to establish retinal topography in the tectum of this species, we began by mapping

Fig. 5. Results of multiunit recordings from the right optic tectum in response to visual stimuli projected onto the back of a translucent hemisphere (case TS2). **A.** The entire tectum dissected from the brain with lesions indicated (red arrows) that were made at selected penetrations sites during the recordings. **B.** Flattened section of the tectum processed for cytochrome oxidase to reveal the lesions (red arrows) in greater detail. **C.** Selected electrode penetrations (circles) with lesions (stars) made during the mapping experiment. **D.** Multiunit receptive fields for neurons recorded at each penetration site at a depth of 50–150 μm. L, lateral; M, medial; R, rostral; C, caudal.


A
Right tectum
L
R
C
M
0.5 mm
B
C
1 2 3 4 5 6 7 8
9 10 11 12 13 14 15
16 17 18 19 20 21 22
Midline
0.5 mm
D
Superior
Inferior
Nasal
Temporal
90 deg.
16 17 18 19 20 21 22
9 10 11 12 13 14 15
1 2 3 4 5 6 7 8

multiunit receptive fields. Fig. 5 illustrates this approach with the tectal anatomy and corresponding receptive field progressions for the right tectum of case TS2. The frontal–nasal receptive fields were represented rostrally in the tectum, and receptive fields moved progressively toward the rear of the animal – temporally – as the electrode was moved more caudally in the tectum. For example, receptive fields for penetrations 1–8 (Fig. 5D) progressed in an orderly, overlapping manner from more nasal to more temporal locations as the electrode location progressed from more rostral to more caudal areas in the tectum (note, however, that numbered penetrations do not always represent a sequence in time during the experiment). Penetrations 9–15 and 16–22 (Fig. 5) illustrate similar nasal to temporal receptive field progressions as the electrode was moved from rostral to caudal in the tectum.

For the medial to lateral axis in the tectum, superior receptive fields were represented most medially (toward the midline) whereas inferior receptive fields were represented laterally. This is illustrated for the multiunit receptive fields in Fig. 5 by the more lateral location of penetrations 1–8 corresponding to the most inferior receptive fields, and by the more medial penetrations 16–22 in the tectum corresponding to the most superior receptive fields, and finally by the middle penetrations 9–15 located in between.

A similar mapping of visual topography in the tectum was observed in subsequent experiments where single unit visual responses were isolated for selected electrode penetrations and multiunit responses to tactile stimulation of the body surface were also recorded at deeper levels (Figs 6 and 7). As has been reported for responses in the tectum

Fig. 6. Results of recordings from the left optic tectum in response to both visual and somatosensory (tactile) stimuli (case TS4). **A.** Schematic diagram of the tectum with electrode penetrations (circles) and lesions (stars) marked. **B.** Receptive fields on the snake's body for multiple units that responded to tactile stimulation at a depth of 400–700 µm. Yellow marks weakly responsive areas. **C.** Multiunit (thin borders) and single unit (thick borders) receptive fields for neurons responding to visual stimuli at each electrode penetration at a depth of 50–150 µm.

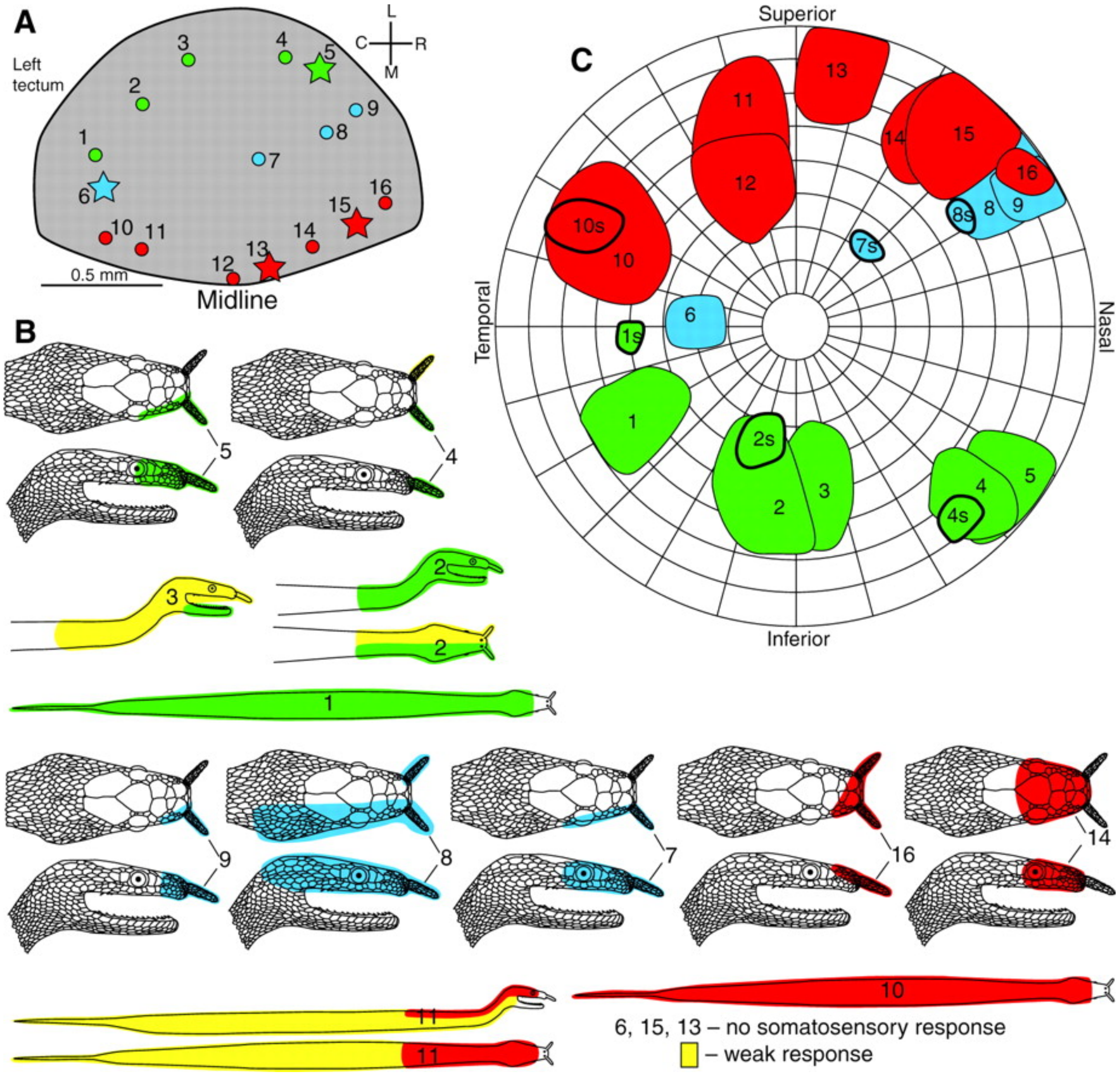
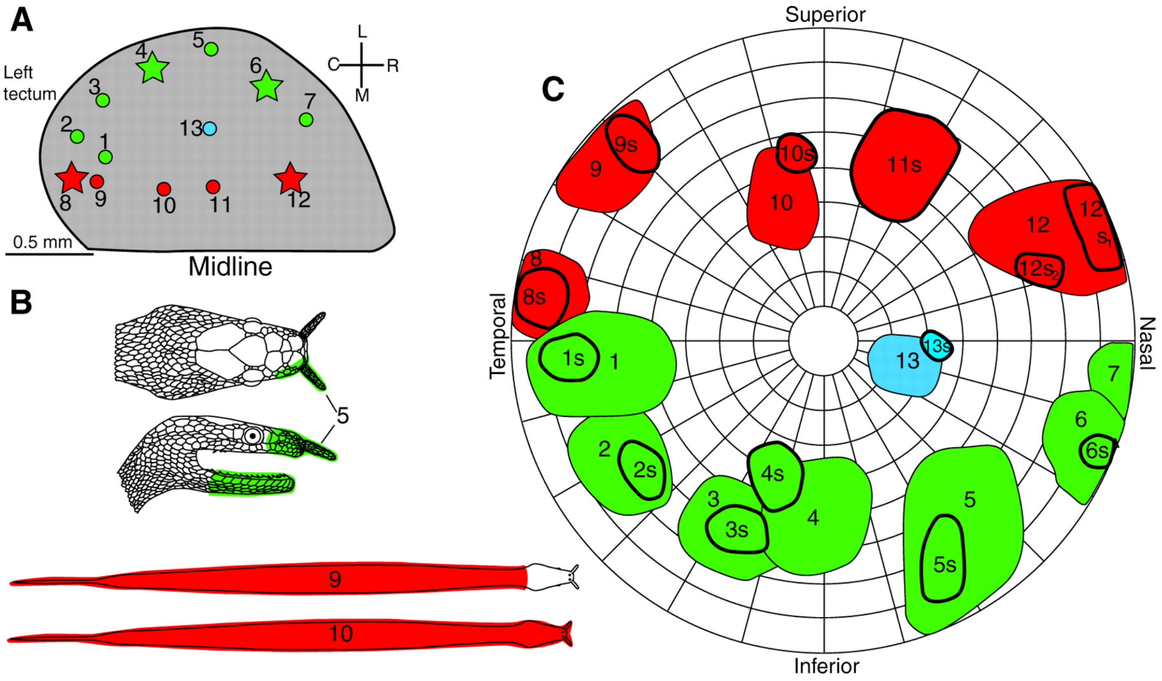

A
Left tectum
L
C
R
M
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
0.5 mm
Midline
B
5
4
3
2
2
1
9
8
7
16
14
11
11
10
6, 15, 13 – no somatosensory response
🟨 – weak response
C
Superior
Inferior
Temporal
Nasal
10s
10
11
12
13
14
15
16
8s
8
9
7s
6
1s
1
2s
2
3
4
5
4s

Fig. 7. Results of recordings from the left optic tectum in response to both visual and somatosensory (tactile) stimuli (case TS5). **A.** Schematic diagram of the tectum with electrode penetrations (circles) and lesions (stars) marked. **B.** Receptive fields on the snake's body for multiple units that responded to tactile stimulation at a depth of 400–700 μm. **C.** Multiunit (thin borders) and single unit (thick borders) receptive fields for neurons responding to visual stimuli at each electrode penetration at a depth of 50–150 μm.


A
Left tectum
L
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Midline
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Superior
Temporal
Nasal
Inferior
9s
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11s
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12 s1
12s2
8
8s
1s
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13
13s
7
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6s
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2s
4s
3
3s
4
5
5s

of other reptiles (Stein and Gaither, 1981), somatosensory responses were weaker and less reliable than visual responses, in part due to fatigue resulting from repeated stimulation of receptive fields. Nevertheless, a number of tactile responses were found in deeper layers, generally at depths of 400 to 700 μm. The overall topography of receptivefields matched the representation of the overlying visual map in the rostro-caudal dimension of the tectum. For example, the tentacle, head and face representation were found predominantly in more rostral to middle tectum (e.g. penetrations 4, 5, 7, 8, 9, 14 and 16 in Fig. 6; penetration 5 in Fig. 7) whereas receptive fields that included the back of the body and did not include the tentacle or front of the face were located in more caudal parts of the tectum (penetrations 1 and 10 in Fig. 6; penetration 9 in Fig. 7). However, receptive fields were often quite large, perhaps because single units were not isolated. A number of receptive fields responded to stimulation of both contralateral and ipsilateral parts of the body. It is possible and perhaps likely that movement of the skin during mapping simultaneously stimulated receptors on both sides of the snake's long, thin body and underside, making precise localization to one side of the body difficult. The orientation of the medial–lateral component of the body map was less obvious, but the few receptive fields found on the lower jaw (penetrations 2 and 3, Fig. 6; penetration 5, Fig. 7) were located in the lateral tectum, whereas penetration 11 in Fig. 6 was in the medial tectum and the strongest response was from the dorsal body surface. This suggested the dorsal–ventral orientation of the body representation was also roughly congruent with the visual representation.

In addition to these stimuli, we illuminated selected visual receptive fields with 850 and 940 nm wavelengths of IR illumination. Neurons in the optic tectum responded to illumination of the receptive field with an 850 nm IR illuminator. The diodes of such illuminators are readily visible to the human eye and, although dim, appear to include shorter, visible wavelengths. Our brief experiment confirmed this possibility, and this was a testament to the sensitivity of the snake's visual system. We also tested 940 nm (covert) illuminators and obtained no visual responses. The latter were subsequently used in our behavioral experiments (below).

Tentacled snake behavior

A number of behavioral observations suggest that tentacled snakes rely heavily on vision. Tentacled snakes exhibited escape responses to visual stimuli and on occasion engaged in a distractive tail wiggling behavior when shadows crossed their visual field. Hungry tentacled snakes could be easily induced to take up their distinctive J-shaped hunting posture by placing a separate container of live fish next to their aquarium. On some occasions, tentacled snakes struck at the movements of an object outside of the aquarium. Finally, when snakes in an enclosure with a transparent bottom were placed on a horizontally positioned flatscreen display, they oriented towards and (after a number of presentations) struck at a simulated fish moving across the screen (supplementary material Movie 1, clip 1). Clearly the tentacles could not play a role in this response. In addition, the responses obtained included the predictive strikes previously reported (Catania, 2009), indicating the snakes had obtained accurate spatial information using visual cues alone.

In addition to trials using only visual cues (the flatscreen fish simulation) we also filmed predatory strikes at fish under 940 nm IR illumination to determine whether tentacled snakes could strike accurately in the complete absence of vision. Although strike frequency was reduced compared with lighted conditions, snakes were able to strike at, and capture, fish without the aid of vision (supplementary material Movie 1, clip 2).

Discussion

Our goal in conducting this investigation was to provide a number of lines of evidence for the function of the tentacles in tentacled snakes (*E. tentaculatus*). Tentacled snakes are fishing specialists, and so it seems reasonable to suppose that these unusual appendages could be used to detect water movements generated by potential prey (Morice, 1875; Winokur, 1977; Smith et al., 2002; Murphy, 2007). However, other possibilities have been suggested (such as a function as lures or in camouflage) and still other functions seemed possible (e.g. a function as electroreceptors or chemoreceptors). To explore these possibilities we examined the anatomy of the tentacles and their innervation, the responses of afferents from the tentacle, and the organization of visual and somatosensory projections to the optic tectum. We also documented snake striking ability with and without visual cues. Some facets of this investigation remain preliminary, and there are many more details to investigate regarding anatomy, electrophysiology and behavior. Nevertheless, the results are telling as regards the function of the tentacles and the senses important to these snakes as they capture fish.

From an anatomical perspective, the tentacles are well-innervated and have characteristics suggesting they would be responsive to disturbances in the water surrounding the head. Specifically, they project into the water providing a lever [like the much smaller cupula on a neuromast (Blaxter and Fuiman, 1989) or sensillae of file snakes (Povel and van der Kooij, 1997)] that remains remarkably flexible and is deflected by the slightest pressure. The terminal innervation is primarily a dense array of fine neurites that cross the center of the tentacle orthogonal to the long axis (Fig. 2B). This internal array of fibers is appropriate for detecting movement and bending of the tentacle itself, but is less appropriate for detecting contact with individual scales, chemical cues, electric fields or thermal cues. In these last cases nerve terminals are typically more superficial in the epidermis (von During and Miller, 1979) or associated with support and sensory cells (Frizsch and Wahnschaffe, 1983).

More direct evidence for a mechanosensory role of the tentacles comes from trigeminal afferent recordings, which showed the tentacles were sensitive to the slightest movement imparted by a 1.65 von Frey hair corresponding to a force of 0.008 g. As would be expected in light of this finding, the afferents also responded to movement of a mechanosensory stimulator in direct contact with the tentacle. More significantly, when the snake's head was submerged in water, afferents supplying the tentacles responded to movement and vibrations of a sphere at a distance of 2 cm.

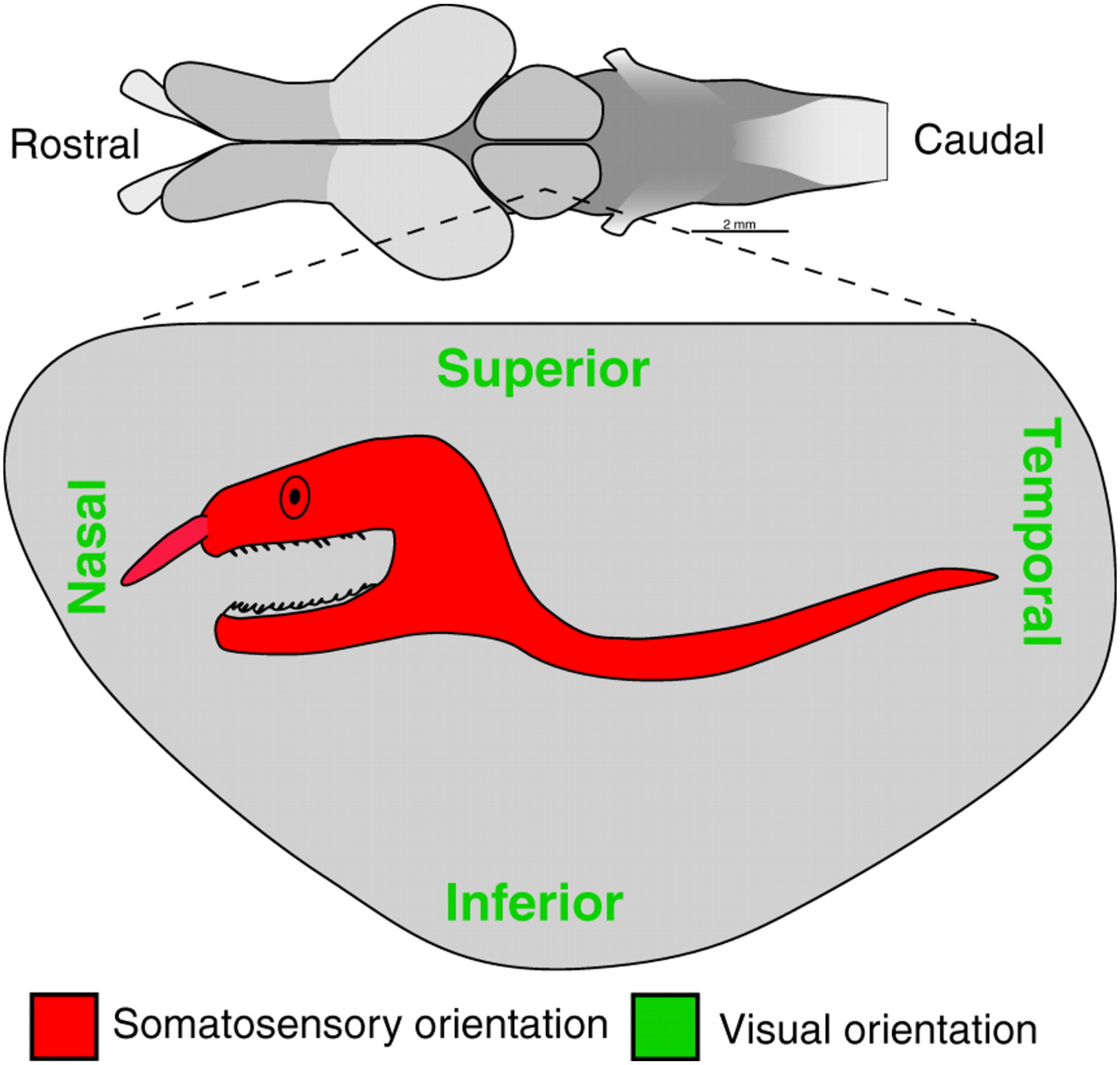
These results suggest the tentacles would be stimulated by prey of the appropriate size and in the favored location for a strike. Tentacled snakes feed on fish and prefer prey that are relatively large compared with their head. They assume a stationary, J-shaped position and usually wait until fish have entered the area between their neck and head to strike. Because this is close to their head (see Catania, 2009) and the head is stationary before a strike, the tentacles are well-positioned to transduce fish generated water movements. A similar function has been shown for whiskers in some semiaquatic mammals that feed on fish (Dehnhardt, 1998; Catania et al., 2008). The densely innervated labial region of the snake's mouth could also contribute prey positional cues based on water movements, as has been demonstrated for alligators (Soares, 2002), which also feed on fish.

The pattern of innervation (Fig. 2C) and lateral view of the head (Fig. 3) suggest the tentacle may be an extension of the larger mechanosensory array on the upper jaw. This possibility is supported by the observation that the labium of snakes in general is densely innervated and in a number of species contains specialized mechanoreceptors (Jackson and Reno, 1975; Jackson, 1977; Jackson and Doetsch, 1977; Jackson and Sharawy, 1980; Westhoff et al., 2005). In addition, boids have adapted densely innervated labial regions for thermal reception (Ross, 1935; Nobel and Schmidt, 1937; Bullock and Barrett, 1968), whereas in crotolines the area corresponding to the tentacles has been modified for thermoreception in the form of pit organs (Noble and Schmidt, 1937; Bullock and Cowles, 1952; Bullock and Fox, 1957). We did not examine potential thermal responses for the tentacles because fish are the same temperature as their environment, and thermal cues could not propagate in water. However, we did test for electroreceptive responses, and none were obtained.

In addition to exploring the tentacles, we were also interested in the visual system, because tentacled snakes seemed to have a well developed eye and optic nerve and their behavior suggested vision plays an important role in guiding strikes. Tentacled snake optic nerves appeared substantial upon dissection (Fig. 2E), comparable in size to the trigeminal nerves. Recordings from the optic tectum revealed vigorous responses to even very dim stimuli projected onto a translucent hemisphere. Receptive fields were topographically organized, with an orientation similar to that reported in mammals and other snakes (Hartline et al., 1978) – and not rotated as reported in Iguana (Gaither and Stein, 1979; Stein and Gaither, 1981). Although fewer responses were obtained for touch, we found that receptive fields on the body, though large, were in approximate register with the overlying visual map (Fig. 8). This suggests that information from mechanoreceptors detecting water disturbances is integrated with visual information in the tectum, much as information from infrared receptors of crotolines (Newman and Hartline, 1981; Kobyashi et al., 1992) and boids (Newman et al., 2004; Molenar, 1992) is integrated with visual information [see also Hartline for vibratory responses in the tectum (Hartline, 1971)]. More generally, the tectum (or superior colliculus in mammals) has a well-established role integrating auditory, visual, and somatosensory information in a range of species (Stein and Meredith, 1993).

Given the emphasis we have put on tentacle function, it seems important to also emphasize that tentacled snakes seem to have a substantial visual system that plays an important role in guiding strikes. This impression was first based on a range of observed visual responses in the course of the study. This was tested explicitly by creating a digital, simulated fish movie that could be played on a horizontally oriented flat screen display. Tentacled snakes that were placed in an enclosure with a clear bottom oriented to the simulation and, after several presentations, struck accurately (in the horizontal

Fig. 8. A schematic drawing of the tentacled snake brain and the superior colliculus with the orientation of the visual (green) and somatosensory maps (red) indicated.


Rostral
Caudal
2 mm
Superior
Nasal
Temporal
Inferior
🟥 Somatosensory orientation
🟩 Visual orientation

dimension) at the simulation (supplementary material Movie 1, clip 1). Thus, tentacled snakes can use vision alone to capture prey. In light of this finding, it was important to conduct the converse experiment. Tentacled snakes were filmed in total darkness except for illumination with 940 nm IR lighting. Although strike frequency was reduced, snakes could strike at and capture fish swimming at a distance of several centimeters from the head and tentacles (supplementary material Movie 1, clip 2). Thus tentacled snakes can also capture fish in the complete absence of vision.

We suggest the tentacled snake's sensory system resembles the situation in barn owls in having two different but important components. When prey are clearly visible, the dominant (Knudsen, 1999) and accurate eyesight of an owl is sufficient to aim a strike. As light levels are reduced (or when prey are concealed), the integration of auditory cues with vision becomes increasingly important and if needed an owl can strike at a sound source in total darkness (Payne, 1971). In the case of tentacled snakes, a relatively large fish swimming close to the head would be clearly visible during daylight. But as light levels are reduced at nightfall (or in turbid water), mechanosensory cues are an important aid for detecting prey and guiding strikes. On the darkest nights and in the most turbid water, the tentacles may allow snakes to continue capturing prey, providing a substantial advantage.

Footnotes

Supplementary material available online at

http://jeb.biologists.org/cgi/content/full/213/3/359/DC1

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CHAPTER IV

Structure, Innervation, and Response Properties of Integumentary Sensory Organs in Crocodilians

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Abstract

Integumentary sensory organs (ISOs) are densely distributed on the jaws of crocodilians and on body scales of members of the families Crocodilidae and Gavialidae. We examined the distribution, anatomy, innervation, and response properties of ISOs on the face and body of crocodilians and documented related behaviors for an alligatorid (*Alligator mississippiensis*) and a crocodylid (*Crocodylus niloticus*). Each of the ISOs (roughly 4000 in *A. mississippiensis* and 9000 in *C. niloticus*) was innervated by networks of afferents supplying multiple different mechanoreceptors. Electrophysiological recordings from the trigeminal ganglion and peripheral nerves were made to isolate single unit receptive fields and to test possible osmoreceptive and electroreceptive functions. Multiple small (<0.1 mm^2) receptive fields, often from a single ISO, were recorded from the premaxilla, rostral dentary, gingivae, and the distal digits. These responded to a median threshold of 0.08 mN. The less densely-innervated caudal margins of the jaws had larger receptive fields (>100 mm^2) and higher thresholds (13.725 mN). Rapidly adapting (RA), slowly adapting type I, and slowly adapting type II responses were identified based on neuronal responses. Several RA units responded maximally to vibrations at 20-35 Hz, consistent with reports of the ISOs' role in detecting prey-generated water surface ripples. Despite crocodilians' armored bodies, the ISOs imparted a mechanical sensitivity exceeding that of primate fingertips. We conclude that crocodilian ISOs have diverse functions, including detection of water movements, indicating when to bite based on direct contact of pursued prey, and in the fine tactile discrimination of items held in the jaws.

Introduction

Crocodilians' faces are covered in arrays of minute, pigmented skin elevations that are clearly visible around the upper and lower jaws. Early anatomical studies revealed differences in their distribution in the three families comprising Crocodilia (von Wettstein, 1937). In Alligatoridae, including the American alligator and caiman species, the protuberances are found only on the head near the mouth whereas in Crocodilidae and Gavialidae, they are found on virtually every scale of the body surface as well as on the head (Figure 1). Despite their prominence, few studies have investigated their function. They have been hypothesized to play a role in the secretion of cleansing and waterproofing oils (Grigg and Gans, 1993), enable osmoreception (Jackson and Brooks, 2007; Jackson et al., 1996), function in mechanotransduction (Necker, 1974), and facilitate courtship behavior (Brazaitis and Watanabe, 2011). Other suggestions include possibly acting as electroreceptors, as a consequence of the aquatic habitat of crocodilians (Bullock, 1999) or acting as magnetoreceptors – an ability noted in alligators (Rodda, 1984). More recently, Soares (2002) discovered that these structures in juvenile *A. mississippiensis* mediate an orienting response to the center of a water surface disturbance. As a result, it was proposed that the main role of these structures is the detection of surface waves generated by prey moving in water, and they were termed "dome pressure receptors" (these organs have been given a variety of names in different studies – we have chosen to follow the functionally neutral and commonly used term "integumentary sensory organ," or ISO (Brazaitis, 1987), in this paper) . Although detection of water disturbances is clearly useful to crocodilians, a number of observations suggest ISOs could facilitate a wider array of mechanosensory abilities. For example the ISOs of crocodylids are found across their entire body and are thus poorly situated for receiving surface waves. In alligators, the highest densities of ISOs are found around the teeth, inside the mouth, and at the rostral margins of the mandibles and maxilla, suggesting a role in discriminating food items or determining appropriate bite force (Erickson et al., 2012). Reflecting this uncertainty regarding their functions, these receptors have also been identified as "integumentary osmoreceptors" in respect to the body receptors found in crocodylids (Jackson and Brooks, 2007).

The goal of this study was to provide further insight into the function of the integumentary sensory organs (ISOs) by comparing them across two species of crocodilians: the Nile crocodile (*Crocodylus niloticus*) and the American alligator (*Alligator mississippiensis*).

Here we examine the anatomy of ISOs, the branching patterns of afferents providing innervation, the physiological response properties of afferents from the skin areas covered with ISOs, and documented some behaviors of alligators and crocodiles capturing prey under infrared illumination and with white noise to mask audition. Our results suggest that both the cranial and body ISOs of juvenile crocodilians are employed as a high resolution mechanosensory system that allows for an otherwise armored skin surface to have a sensitivity greater than primate fingertips.

Materials and Methods

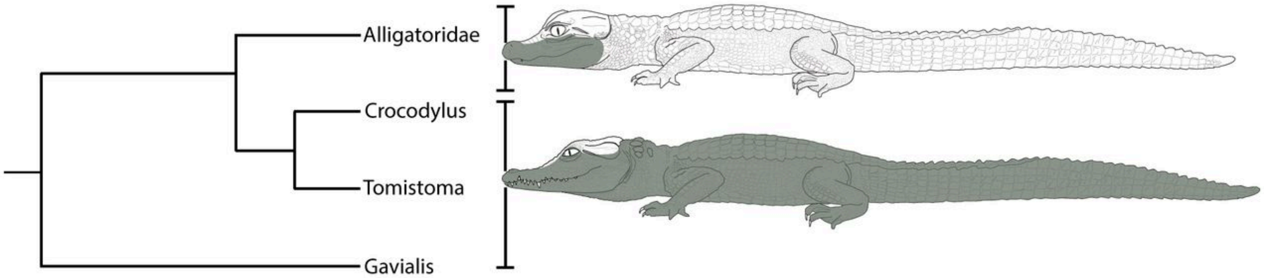
Animals

Eighteen American alligators (*Alligator mississippiensis* Daudin 1801) and four Nile crocodiles (*Crocodylus niloticus* Laurenti 1768) were studied. The alligators were provided by the Department of Wildlife and Fisheries from the Rockefeller Wildlife Refuge (Grand Chenier, LA, USA), and Nile crocodiles were purchased from a commercial reptile breeder (Brooksville, FL, USA). They ranged in total body length from 15 to 92 cm and in mass from 30 g to 3.2 kg (from newly-hatched to approximately 3 years).

Scanning electron microscopy

Animals were sacrificed with sodium pentobarbital (120 mgkg^{-1}) and perfused with 4% paraformaldehyde (PFA). Tissues from the head and body surface were immersion fixed for 24 to 48 hours, rinsed with phosphate-buffered saline (PBS),

Fig. 1. The phylogeny of extant crocodilians, as modified from (Brochu, 2003). On the left, the distribution of integumentary sense organs (ISOs) is indicated by the shaded regions. Within the order Alligatoridae, which includes all *Caiman* and *Alligator* species, ISOs are restricted to cranial regions. Within the *Crocodylus*, *Tomistoma,* and *Gavialias* genera, ISOs are located on the cranium as well as along the rest of the post-cranial integument. Debate continues on the phylogenetic relationship of *Tomistoma* to *Gavialis*, depending on the genomic materials used in analysis (Piras et al., 2010).


Alligatoridae
Crocodylus
Tomistoma
Gavialis

and dehydrated in a graded series of ethanol. Following dehydration, samples were critically-point dried in an E3000 drier (Quorum Technologies, Guelph, ON, Canada) and coated with gold in a Cressington 108 sputter coater (Cressington Scientific Instruments Ltd, Watford, UK). Specimens were imaged using a Tescan Vega II SEM (Tescan, Cranberry Twp, PA, USA).

Sudan Black B

Specimens were fixed in 4% paraformaldehyde for at least 1 week, washed in tap water for 12 hours, and then cleared in 10% hydrogen peroxide for 2 to 3 days. Following washes in deionized water, maceration in trypsin solution, and washing in potassium hydroxide, the samples were stained in Sudan Black B solution (0.5 g Sudan Black B, Sigma Chemical Co, St. Louis, MO, USA). Specimens were destained in ethanol and preserved in glycerin.

Receptor density measurements

Surfaces of the heads from two alligators (SVL = 46 cm) were photographed by incrementally rotating the samples. Individual photographs were aligned based on distinguishing landmarks to create a complete montage of the scaled surfaces of the dorsal and ventral surfaces of the upper and lower jaws, and inside the oral cavity. A grid of 36 squares, each 2 mm x 2mm, was superimposed on the completed montages. The number of receptors within each box was counted, excluding the top and left walls, and the distance between individual receptors was measured using ImageJ (National Institutes of Health, Bethesda, MD, USA). Results from the 4 hemispheres were averaged.

DiI and confocal microscopy

Scale surface samples were removed post-mortem from paraformaldehyde-fixed tissues. Small crystals of DiI (1, 1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; Molecular Probes, Invitrogen, Carlsbad, CA, USA) were applied with insect pins to the exposed branches of the maxillary and mandibular nerves innervating facial regions and to the intercostal nerves for the ventral body surface. The scales were embedded in 2% agarose, immersed in 4% PFA, and stored in darkness for approximately 1 week. The specimens were sectioned sagittally on a Vibratome Series1000 (Technical Products International, St. Louis, MO, USA) and imaged on an upright LSM510 confocal microscope (Zeiss, Thornwood, NY, USA).

Trigeminal nerve light microscopy

Segments of the ophthalmic, maxillary, and mandibular branches of the trigeminal nerve from 3 age-matched yearling alligators and 2 Nile crocodiles, approximately 2 years old, were dissected following perfusions with 4% PFA. Tissue was sampled 2 to 4 mm from the body of the trigeminal ganglion, and specimens were immersed in phosphate-buffered 2.5% glutaraldehyde solution for least 24 hours. Samples were post-fixed in osmium tetroxide, dehydrated in a graded ethanol series, transferred into propylene oxide, and embedded in EMBed 812 (EM Sciences, Hatfield, PA, USA). Samples were sectioned transversely at approximately 0.5 μm thickness using a diamond knife (Diatome US, Hatfield, PA, USA) on a Reichert Ultracut E ultramicrotome. Tissue was examined at 100X under light microscopy (Zeiss Axioskop, Zeiss, Jena, Germany), and digital images were captured (Axiovision 4.5) and compiled in Adobe Photoshop

CS5 (Adobe Systems, San Jose, CA, USA) into complete montages of the transverse section of the nerve of interest. Myelinated axons were manually counted.

Trigeminal and peripheral responses

Eighteen alligators and two Nile crocodiles were anesthetized with a combination of urethane (0.4 gkg^{-1}), ketamine (100 $mgkg^{-1}$), and xylazine (20 $mgkg^{-1}$). Supplemental doses were given as needed. The trigeminal ganglion ipsilateral to the stimulated body surface was exposed. For recordings from the body integument, the radial and ulnar nerves were exposed in the proximal regions of the forelimb, and the median nerve was exposed in the hindlimb. Receptive fields were marked on photographs of the body. Multi-unit and single-unit electrode recordings were made approximately 400 to 800 µm from the ganglion's surface using tungsten electrodes (1.0 to 1.5 MΩ at 1000 Hz). Responses were collected using a Bak headstage and preamplifier (BAK Electronics, Inc., Mt. Airy, MD, USA) and sent to a Neurolog amplifier and filters (Digitimer, Welwyn Garden City, Herts, UK). Responses were monitored using speakers and waveforms from single units were collected at 100,000 samples/s using Labchart 7.0 software using a Powerlab 4/30 system (ADInstruments, Colorado Springs, CO, USA) attached to a MacBook laptop (Apple, Cupertino, CA, USA). The skin surface was kept moist during recordings.

Several combinations of somatosensory stimuli were used to elicit responses from the skin surface. Scales were examined with small wooden probes and von Frey filaments (Stoelting Company, Wood Dale, IL, USA). Filaments just beyond threshold for eliciting a response were used in detailing the borders of receptive fields on photographs of the skin surface. Using the Chubbuck stimulator (Chubbuck, 1966) and the digital sine and square wave generator in Labchart 7.0, the frequency of the tactile stimuli was systemically altered. The motion of the stimulator was recorded to observe the timing of responses. Other stimuli included various positions of pair of 9V batteries and room temperature hypertonic salt solutions (47 parts per thousand or greater) using Instant Ocean sea salts (Aquarium Systems, Mentor, OH, USA).

Following recordings, selected trigeminal ganglion electrode penetrations were lesioned with a 10 µA current for 15 seconds while the electrode was withdrawn from the ganglion at 50 µm/second; other selected penetrations were marked with toluidine blue stain. Crocodilians were given an overdose of pentobarbital and perfused with PFA, as described above. Images of the intact ganglion were matched with photographs marked with locations of electrode penetrations. The results from 6 alligators' trigeminal ganglia were used to reconstruct the somatotopy of the ganglion. Measurements of receptive field size were made using ImageJ (National Institutes of Health, Bethesda, MD, USA).

Single unit recordings (n=110) from 4 alligators' trigeminal ganglia were used to assess characteristics of receptor-covered skin. Data collected through LabChart 7.0 was analyzed using the Spike Histogram module to measure the interspike interval between consecutive action potentials. This was measured in the static phase (200 to 500 msec) after the dynamic response to initial stimulus presentation.

To assess differences between the von Frey force thresholds, the surface area of receptive fields, and the location of the field (from either crocodile or alligator and from the cranial or post-cranial body scales from both), a series of independently-sampled ttests was run.

Pearson correlation coefficients and Spearman's rank correlation coefficient were calculated, as the force thresholds represented a discontinuous data set whereas receptive field surface areas were continuous. All statistical analyses were two-tailed and set at the 0.05 level of significance. These were completed using JMP Version 9.0 (SAS Institute, Cary, NC, USA).

Behavior

Nile crocodiles and American alligators were filmed with a MotionPro HS-3 camera with video recorded on a MacBook pro computer running MotionProX software (Redlake, Integrated Design Tools,Talhassee, FL, USA). Animals were placed in aquaria with room temperature water and permitted to move freely. Infrared lighting was provided on indicated trials with two IR-Flood Ultra-Covert 940 nm illuminators (Night Vision Experts, Buffalo, NY, USA). White noise was presented on indicated trials as generated in Audacity (Carnegie Mellon Computer Music Group, Pittsburgh, PA, USA). Video was analyzed using iMovie (Apple, Cupertino, CA, USA). All procedures conformed to the National Institutes of Health standards concerning the use and welfare of experimental animals and were approved by the Vanderbilt University Animal Care and Use Committee.

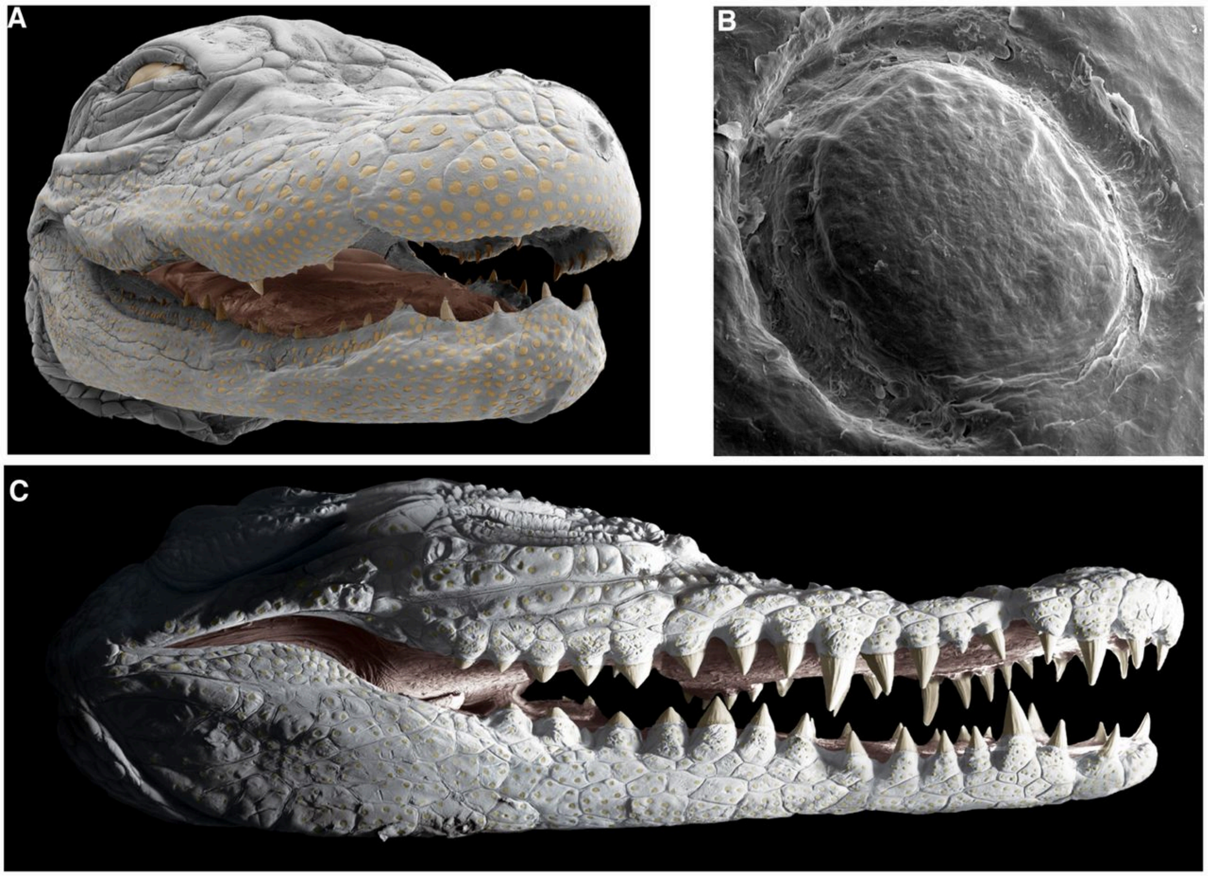
Results

In examining the crocodilian integumentary sensory organs (ISOs), several levels of analysis were adopted for both alligatorids and crocodylids. These data begin with describing the distribution of the organs, then their structure and innervation, and next, the trigeminal and spinal afferent electrophysiological responses recorded from the stimulation of skin and individual ISOs. Finally, some behavioral observations of the animals orienting towards food pellets and live prey are noted in reference to Movie 1 in the supplementary materials.

ISO distribution

Figure 2A shows the head of a juvenile American alligator. Skin on the dorsal and ventral areas was covered in small, elevated sensory organs (Fig. 2B). In each of the three juvenile alligators examined which included a single year old alligator (head length = 7.2 cm) and two approximately 3 year old alligators (head length = 15.0 cm), there were approximately 4200 ISOs (Std. deviation = 94) distributed across the cranial regions. In the same areas on two juvenile Nile crocodiles (Fig. 2C), there were 3001 and 2811 ISOs. These cranial ISOs varied in size across the facial surface, with the smallest receptors found in apposition to and between the teeth and the largest receptors located on the dorsal surface of the maxilla, with mean diameters of 0.2 (Std. deviation=0.03mm) to 1.2 mm (Std. deviation = 0.04mm) respectively. Within the oral cavity, the ISOs were distributed across the upper palate and the gingivae near the tongue. In alligators the

Fig. 2. Crocodilian cranial regions viewed under the scanning electron microscope. **A.** The colorized head of *Alligator mississippiensis* hatchling. The ISOs (colored in yellow) are visible as circular, dome-shaped elevations. **B.** A single *Alligator* ISO is shown in higher magnification, with a hinge region surrounding the elevated central region of the ISO. **C.** The cranial regions from a *Crocodylus niloticus* juvenile, showing the distribution of ISOs.


A
B
C

greatest concentration of ISOs (> 2.00 receptors/mm^2 on a 15 cm head) was surrounding the teeth, and lower densities were found on the dorsal maxilla (Fig. 3). The distance between ISOs ranged from 0.3 mm (Std. deviation=0.09 mm) in the areas surrounding the incisor teeth on the rostral dentary to 4.9 mm (Std. deviation=0.13 mm) on the dorsal surface of the maxilla. For the anterior-posterior axis, ISO density was greater near the most anterior point, particularly on the lower jaw. Interestingly, disjunct areas of greater ISO density were found directly ventral to the eye and surrounding the nares. No evidence was found for other receptor organs (e.g., ampullary organs, ciliated receptors).

The post-cranial receptors of crocodylids were less densely distributed but found across the entire integument, including on the armored post-nuchal scales and osteoderms on the dorsal surface. Similar to the cranial receptors, they were visible as small, pigmented protuberances; however, there was typically only one ISO located caudally on each scale (occasionally as many as 2 to 3). As a result, they were densest where the scales themselves were small, most notably near the cloaca.

ISO structure and innervation

Below the outer keratinized layers of epidermis of each ISO, a diversity of mechanoreceptors was positioned just beneath the stratum spinosum, supplied by a network of myelinated and unmyelinated axons. Transverse sections from the receptor revealed a number of anatomically distinct endings of the innervating axons (Fig. 4). The connective tissue below each receptor contained many melanocytes and provided the ISOs with their distinctive pigmentation. Just below the stratum basale, the melanocytes branched extensively and were filled with darkly-colored melanin granules. These

Fig. 3. Density of the ISOs across the cranium of juvenile *Alligator*. Density was greatest directly adjacent to the teeth and near the rostral-most points of the maxilla and dentary. Isolated patches of greater density were found surrounding the nares and below the eye.

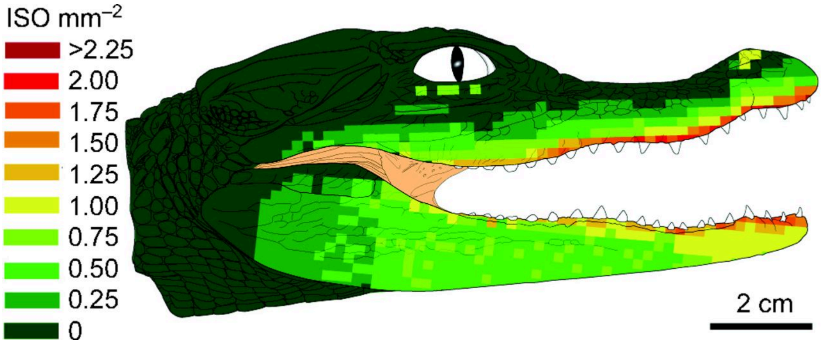
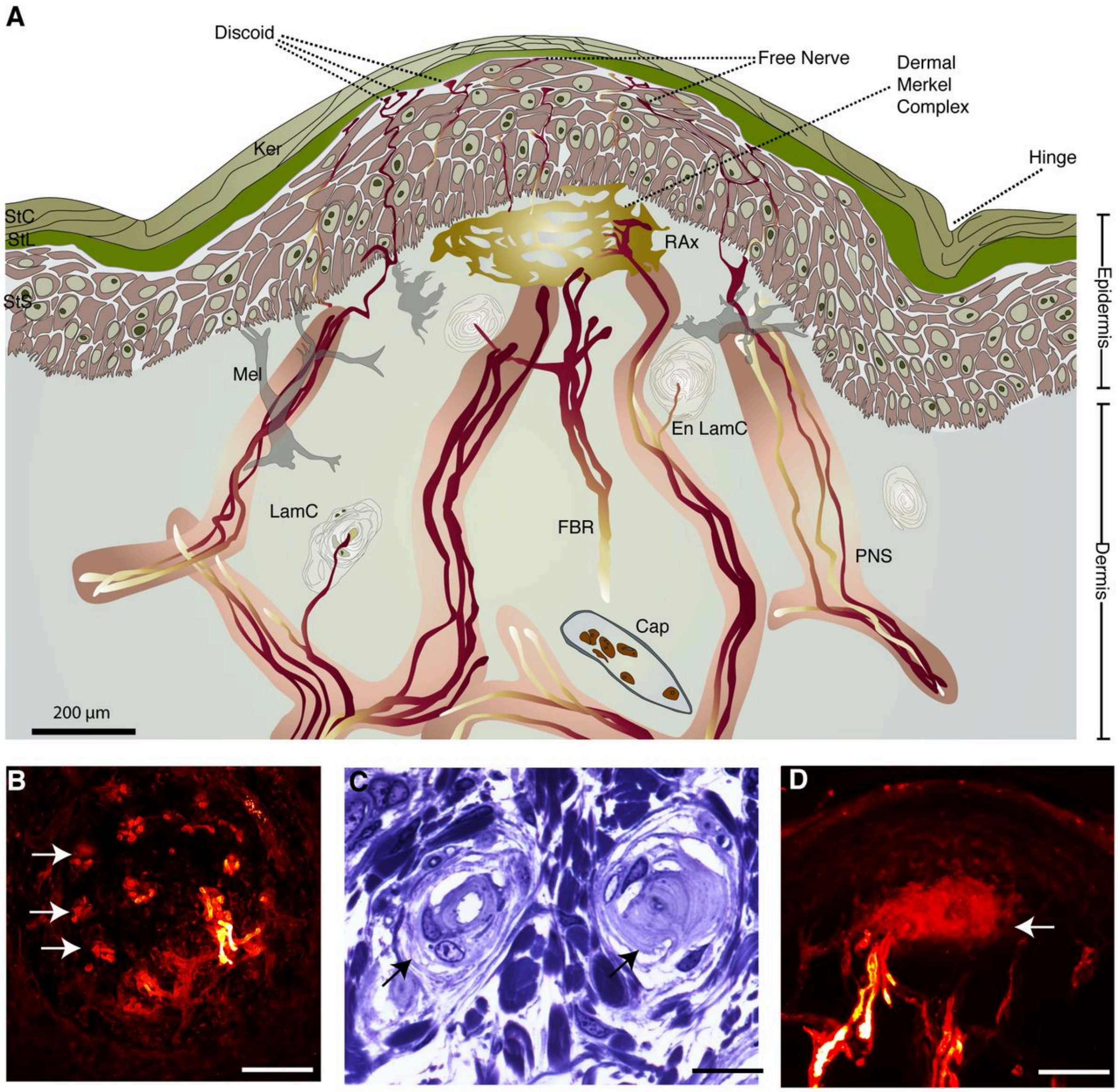

ISO mm⁻²
>2.25
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0
2 cm

Fig. 4. The structure of the crocodilian ISO. **A.** Schematic representation based on samples from *A. mississippiensis* and *C. niloticus* cranial and body receptors. A diverse array of tactile components was localized to the epidermis and dermis of the ISO. Discoid receptors with enlarged terminals and free nerve endings ran through the keratinized epidermal layers that overlaid the prominent dermal Merkel complex and large branching network of myelinated axons. **B.** Confocal fluorescence of DiI-labeled free nerve endings (arrows) from a section tangential to the surface of the receptor, at 20 µm below the apex. Scale bar is 10 µm. **C.** Lamellated corpuscles (arrows) were visible in toluidine-blue stained sagittal sections from the dermis of the receptor. Scale bar is 50 µm. **D.** A large dermal Merkel complex (arrow) and related branches of DiI-labeled nerve fibers as seen under confocal microscopy in a cross section. Scale bar is 200 µm. Cap, capillary; discoid, discoid receptor; En LamC, encapsulated lamellated corpuscle; FBR, free branched receptor of the connective tissue; ker, β-keratinocyte; LamC, lamellated corpuscle; Mel, melanocyte; PNS, perineural sheath; RAx, branched receptor axons of the ISO connective tissue; StC, stratum corneum; StL, stratum lucidum; StrS, stratum spinosum.


A
Discoid
Free Nerve
Dermal
Merkel
Complex
Hinge
Ker
StC
StL
StS
RAx
Mel
En LamC
LamC
FBR
PNS
Cap
200 µm
Epidermis
Dermis
B
C
D

granules were interspersed with the mechanoreceptors. Unmyelinated free nerve endings, approximately 0.5 µm (S.D. =0.09 µm) in diameter, passed through the connective tissue layers and terminated in the outer stratum spinosum. Branching from larger bundles of myelinated axons, free intraepidermal terminals were visible as ubiquitous "discoid receptors" and could be distinguished based on their rounded, expanded structure located just below the cells of the stratum lucidum and corneum. Discoid receptors were closely coupled to the tonofibrillar structures of the adjacent cells of the spinosum and lucidum, and fluorescent, lipophilic dye applied to the proximal ends of the myelinated bundles often labeled the keratinized cells of the stratum corneum. The extracellular space between individual stratum spinosum cells was compressed at the point of receptor termination where the discoid receptors were located and surrounded by the tonofibrils of individual neighboring cells. Reflecting this compression, the keratinized layers of the stratified epithelium directly over the ISOs were approximately 60% thinner than that of adjacent scaled regions (n=24, S.D = 27 µm). The most superficial of the keratinized layers, the stratum corneum, appeared thinnest in the domed receptor region and at the hinged region of epidermal folds between individual scales.

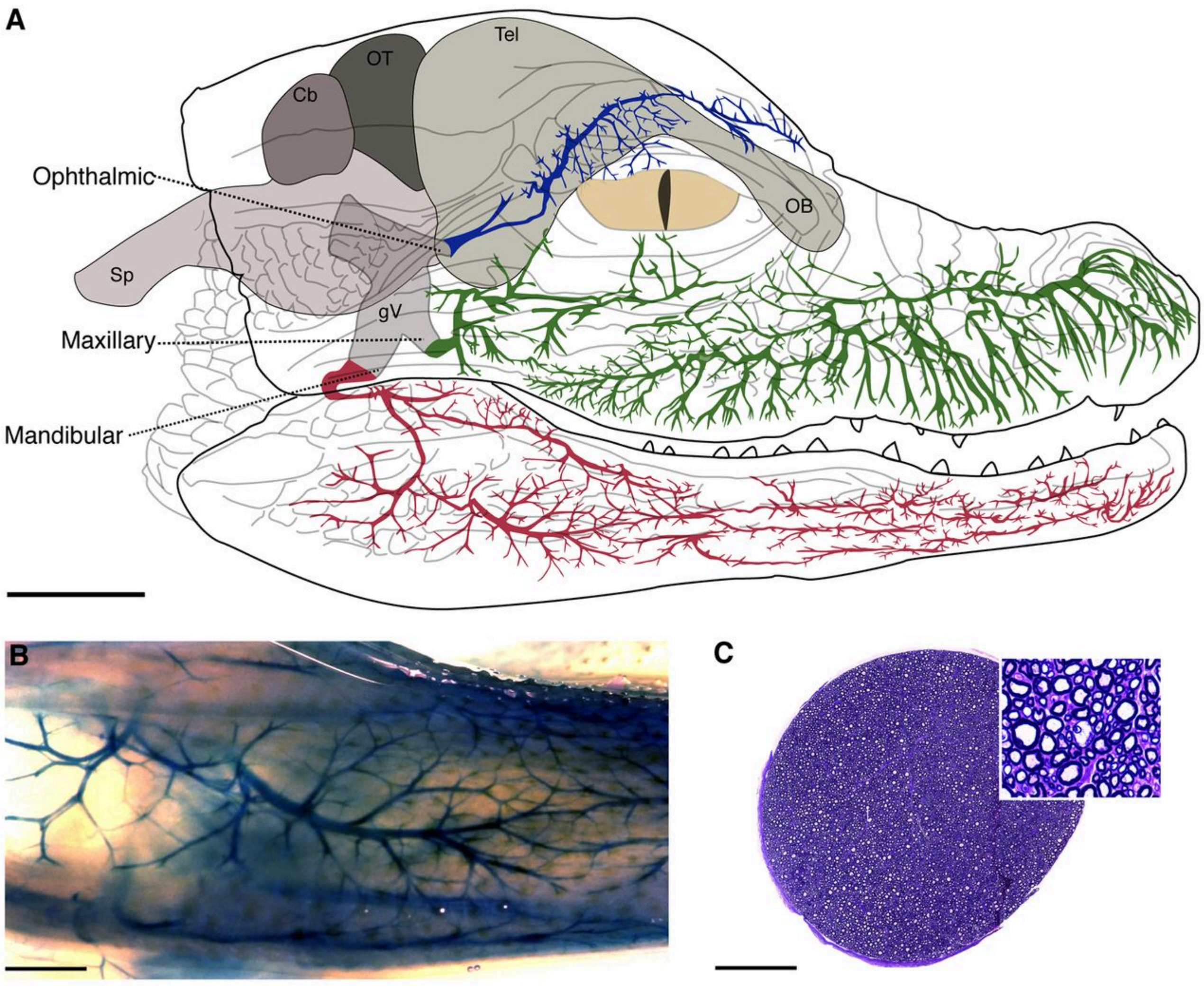
Within the connective tissue beneath each receptor numerous axon terminals were ensheathed in lamellations of Schwann cell processes (Fig. 4C). They appeared similar to the Paciniform corpuscles found in mammalian skin (Pease and Quilliam, 1957). There were also mechanoreceptor components that were not affiliated with Schwann cell elaborations. These included free axon terminals running parallel to the collagen fibers and ending in the dermis, with the morphology of previously identified branched lanceolate terminals (von Düring and Miller, 1979).

The most prominent sensory nerve endings of the ISOs' were associated with the dermal Merkel cell column, located below the center of the dome and the surrounding stratum spinosum, where many of the axons traversing the longitudinal axis between domes converged (Fig. 4D). A similar configuration has been described in *Caiman* (von Düring, 1974). This structure was easily distinguished as a mass of elongated, flattened Merkel cells with intercalated axon terminals and was distinct in its limited distribution to regions under the ISOs.

The ISOs were supplied by fibers that originated below the superficial layers of the collagenous tissue from an elaborate network of myelinated fibers that ran parallel to the skin surface. These branched most distally at the dome receptor regions into fascicles that typically contained 15 or more myelinated axons (arrowhead in Fig. 4B). At more superficial levels, these branches coursed together in circular patterns, ringing the inner circumference of the dome when viewed in horizontal sections, with nerve endings branching from larger groupings. At lower levels, the myelinated bundles were markedly larger in diameter (75 µm, S.D. = 13 µm).

Cleared specimens stained with Sudan Black B revealed the origin of the nerves in the trigeminal system (Fig. 5). This preparation was useful for identifying the large rami of the trigeminal nerve and for following the finer terminals to areas covered in ISOs (Fig. 5B). These data are shown in the schematic created from 2 hatchling alligators. A stained specimen from a juvenile Nile crocodile showed a similar pattern of innervation. The trigeminal nerve bifurcated into the mandibular and maxillary/ophthalmic lobes approximately 1 to 2 mm from the Gasserian ganglion. The mandibular nerve then ran through the external mandibular fenestra and extended both

Fig. 5. Innervation of the cranial ISOs by the trigeminal nerve. **A.** Side view of the rami of the trigeminal nerve with hypertrophied mandibular and maxillary branches comprising a network of finer fibers innervating regions where ISOs are present. Branching patterns were drawn from Sudan Black B preparations (see Materials and methods). The brain is shown to indicate the relative location of the trigeminal ganglion. Scale bar is 1 cm. **B.** Example photograph of Sudan Black B preparation showing the darkly-stained processes of the maxillary nerve within the cleared whole-mounted specimen. Scale bar is 750 µm. **C.** Transverse section of a mandibular nerve from *C. niloticus*. More than 46000 myelinated axons (Std. deviation = 2700), as seen in the inset, were present within the nerve whereas fewer (3600; Std. deviation = 200) were present in the ophthalmic component. Scale bar is 50 µm. Cb, cerebellum; gV, trigeminal ganglion; OB, olfactory bulb; OT, optic tectum; Sp, spinal cord; Tel, telencephalon.


A
Tel
OT
Cb
Ophthalmic
OB
Sp
gV
Maxillary
Mandibular
B
C

caudally to the back of the angular bone and rostrally to the teeth and anterior portions of the dentary. From the fenestra, the mandibular ramus branched extensively into at least three smaller ramules running parallel to the mandible and narrowed as it extended rostrally. The maxillary ramus ran from the jugal and quadratojugal and appeared equally diverse in its arborization near the ISOs. Both the dentary and the maxilla had many small foramina, and nerve fibers ran through the bone to project out of these openings in both directions on the rostrocaudal axis. Typically, the afferents of a single cranial foramen innervated 3 ISOs. In addition to supplying fibers to the external surface of the jaws, both the mandibular and maxillary rami innervated the palate and gingivae extensively, both of which were covered in ISOs.

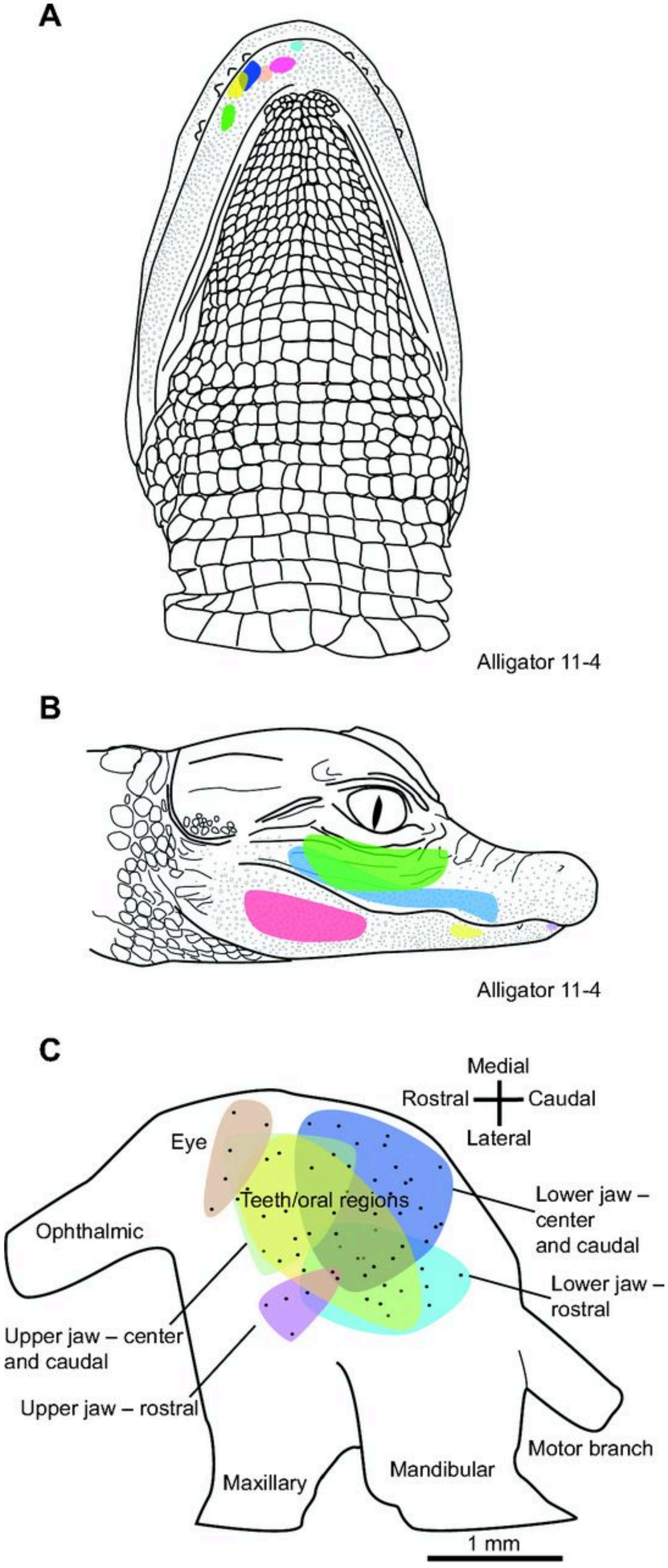
The ophthalmic ramus, which mainly innervated the largely receptor-free nasal and lacrimal bone areas as well as the dorsomedial surface of the cranium, was much smaller than the mandibular and maxillary rami and did not branch extensively. In 4 yearling alligators, the mandibular and maxillary rami contained approximately 46500 (Std. dev = 2700) and 48300 myelinated axons (Std. dev = 3300) whereas the ophthalmic ramus contained only 3600 myelinated fibers (Std. dev =200). Similarly, in 4 Nile crocodiles matched in age and body size to the alligators, there were approximately 46300 myelinated axons (Std. dev = 2800) in the mandibular, 49400 (Std. dev =3000) in the maxillary, and 3300 (Std. dev = 300) in the ophthalmic rami.

Responses of neurons in the trigeminal ganglion

The trigeminal ganglion was found ventral to the ear, behind the jugal in anatomical dissections. To examine the responses of the cranial ISOs, we recorded extracellular activity from afferent cell bodies in the ganglion. We began by characterizing the location and size of receptive fields corresponding to multiunit activity elicited by stimulating the skin with fine wooden probes and calibrated von Frey hairs. Receptive fields were found for the majority of the skin surfaces across the face of each crocodilian, including areas on the external surface of the mandible, the maxilla, the jugal bones ventral to the eye, and within the oral cavity, among other regions. The extent of these multiunit fields was documented and then single units were isolated to investigate individual afferents in greater detail.

The majority of receptive fields corresponded to skin areas covered in ISOs (Fig. 6). Large receptive fields were found across the jaws and often extended ventrally across the mandible or dorsally to areas near the nares on the maxilla. Although some receptive fields were located on facial areas where ISOs were absent (i.e., the skin area dorsal to the suprangular and ventral to the quadratojugal), the majority of responses were elicited by stimulation on or near ISO-covered skin. Furthermore, receptive fields were organized in an overlapping manner, with the same area of ISOs often represented in two different locations in the ganglion. Ganglion cells responsive to mechanical stimulation near the eye were located rostromedially near the ophthalmic branch whereas cells responsive to mechanical stimulation of the upper and lower jaws were found more caudally in the ganglion. A large area of the ganglion between the maxillary and mandibular branches contained cells that responded to stimulation of the teeth, upper

Fig. 6. Representations in the trigeminal ganglion. **A.** Ventral view of the lower jaw with representative receptive fields which were often small and near the rostral margins of the head. **B.** Side view from the same case, showing the larger, overlapping fields that are characteristic of the more caudal regions of the dentary and maxilla. **C.** Composite figure from 10 *A. mississippiensis* and 2 *C. niloticus* trigeminal recording cases. Large regions representing the teeth and mouth are present on the center of the body of the trigeminal ganglion, while a smaller region located rostrally contains neurons responding to the ISO-sparse areas near the eye.


A
Alligator 11-4
B
Alligator 11-4
C
Medial
Rostral
Caudal
Lateral
Eye
Teeth/oral regions
Ophthalmic
Lower jaw – center and caudal
Lower jaw – rostral
Upper jaw – center and caudal
Upper jaw – rostral
Maxillary
Mandibular
Motor branch
1 mm

palate, and tongue (Fig. 6C).

In total, 216 single units from the trigeminal ganglion were recorded and analyzed in juvenile alligators, and 53 were examined in Nile crocodiles. Their receptive fields were plotted on photographs taken of each individual crocodilian. In general, the tactile receptive fields corresponding to the most rostral regions of the animal's face were smallest and more numerous compared to those corresponding to caudal regions innervated by the mandibular and maxillary nerves (Fig.6 A, B). The smallest receptive fields encompassed single ISOs, with surface areas of less than 1 mm^2. These fields comprised of a single ISO were most often (92%) found near the rostral aspect of jaws, though a few were found on more caudal regions of the face. Larger cranial receptive fields contained more than 240 ISOs and were as much as 130 mm^2 in area (not illustrated).

After recording the area of each receptive field, mechanosensory thresholds were measured using calibrated von Frey hairs. We found that wet skin surfaces provided lower thresholds compared to dry skin, and thus all recorded data came from preparations with moisture maintained. Among the 174 single unit receptive fields measured for indentation force, results ranged from 13.725 mN to 0.078 mN, corresponding to von Frey filaments numbered 4.17 to 1.65 respectively. The lowest threshold could not be established for the 28 receptive fields that were sensitive to the 1.65 filament as this was the smallest calibrated force that could be applied. The most sensitive areas were concentrated near the rostral premaxilla and mandible, as well as in apposition to the teeth. All of the receptive fields that were restricted to a single ISO were responsive to the 0.078 mN (smallest) indentation force. Afferents with the highest thresholds were generally found near the relatively sparsely-innervated regions on the dorsal surface of the maxillary, between the nares and the eyes, and at the caudal margins the jaws. In general, afferents that were activated by the least pressure had small receptive fields whereas afferents responding to the stimulation of many ISOs (large receptive fields) required greater force (13.725 mN or more).

To investigate the responses of afferents to precisely controlled stimuli, we employed a dedicated mechanosensory stimulator (Chubbuck, 1966). The Chubbuck stimulator was driven by a sine wave or square wave generator that controlled the linear movement of a small probe in a single dimension. The probe's location was precisely tracked by a calibrated analogue output of the stimulator (Fig. 7).

Afferents that responded to the onset and offset of square-wave stimuli were characterized as rapidly adapting (RA) (Fig. 7E,D and Fig. 8) whereas afferents that responded throughout the duration of the stimulus were characterized as slowly-adapting (SA) (Fig. 7A, D, F). The SA responses could be further subdivided into SA I and SA II, based on the coefficient of variation (CoV) of the interspike interval (ISI) during the static phase (200 to 500 msec) of the maintained stimulus. This was calculated as the standard deviation of the ISI divided by the mean ISI for the 2 second train of action potentials (Chambers et al., 1972; Wellnitz et al., 2010). SA I units displayed irregularly timed discharges in response to the maintained stimulus whereas SA II units had regularly timed discharges during the same period. Among 110 units in 4 alligators, 51 % of the responses were RA and 49 % were SA (Table 1). Of the slowly-adapting responses, 39% were SA I and 37 % were SA II. The remaining 24% of the SA

Fig 7. Responses of trigeminal afferents from ISO-covered skin in crocodilians. **A.** The small receptive field was located on the juvenile *Alligator*. **B.** Recording of the movement of the stimulator based on a calibrated analogue output proportional to displacement. **C.** Response of the afferent to the displacements shown in (B) showing the discharge pattern of a typical SA type II. The interspike interval increased monotonically with increased displacement amplitude, maintaining the 'regular' firing pattern indicative of SA type II fibers. **D.** SA (in this case type I) and RA responses were present for receptive fields covering individual ISOs near the teeth in this *Alligator* case. In parts D through F, the output of the stimulator is recorded below the response of the afferent. **E.** A RA unit from a juvenile *C. niloticus* is shown responding to block and sinusoidal (20 Hz) stimuli. The photograph of the crocodile has been reversed to show the small receptive field more clearly. **F.** Larger receptive fields, covering multiple ISOs, were found at the caudal margins of the jaws as illustrated in an *Alligator* case.

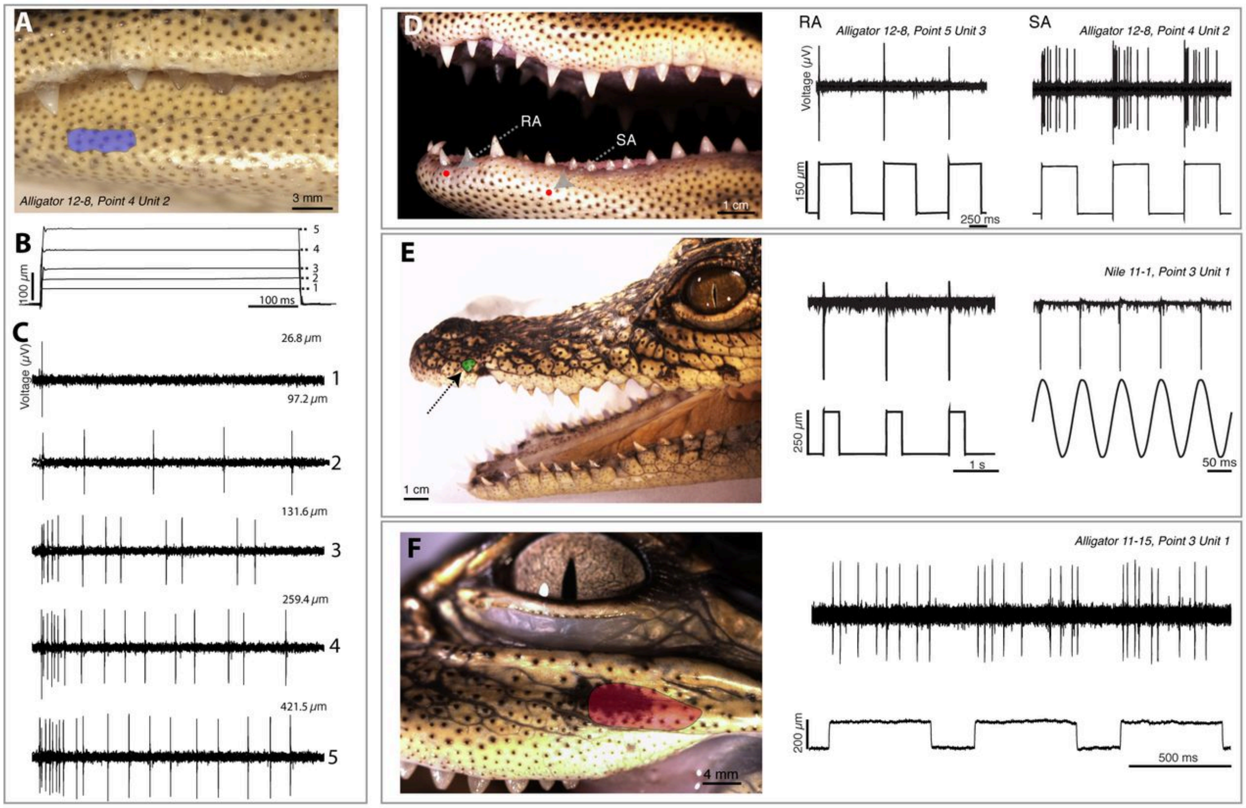
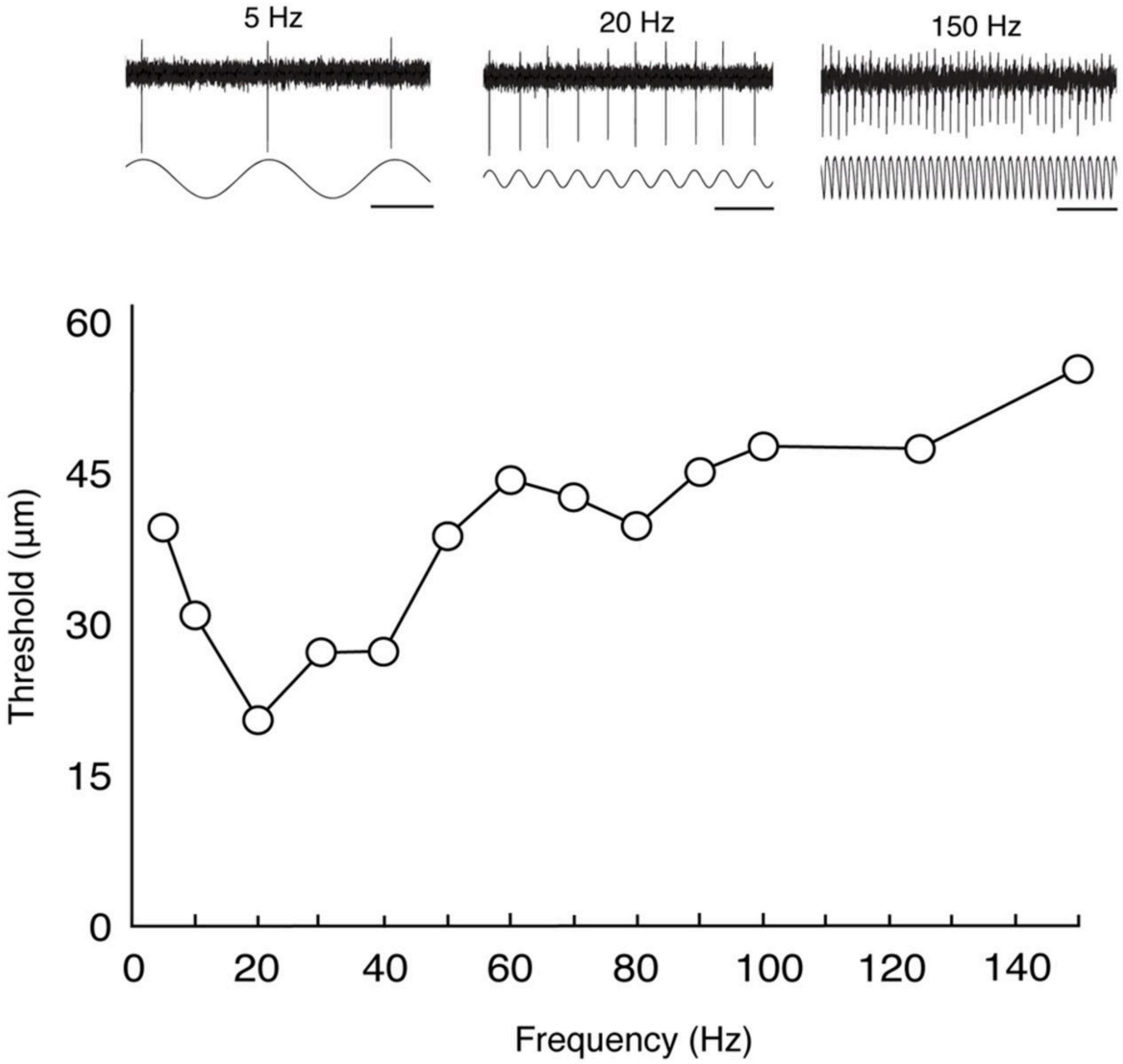

A
Alligator 12-8, Point 4 Unit 2
3 mm
B
100 μm
100 ms
5
4
3
2
1
C
Voltage (μV)
26.8 μm
1
97.2 μm
2
131.6 μm
3
259.4 μm
4
421.5 μm
5
D
RA
SA
1 cm
RA
Alligator 12-8, Point 5 Unit 3
SA
Alligator 12-8, Point 4 Unit 2
Voltage (μV)
150 μm
250 ms
E
1 cm
Nile 11-1, Point 3 Unit 1
250 μm
1 s
50 ms
F
4 mm
Alligator 11-15, Point 3 Unit 1
200 μm
500 ms

Fig 8. Tuning curve for an *Alligator* cranial ISO. Upper panels: Recordings of 1:1 entrained responses from one trigeminal *Alligator* RA unit to sinusoidal stimulation of increasing frequency. The movement of the stimulator is illustrated below each afferent recording. Scale bar is 100 msec. Lower panel: The threshold displacement of the probe required to produce 1:1 entrained responses for a single afferent from 10 to 150 Hz. Thresholds were lowest in the 20-30 Hz range and were greater with both lower and higher frequencies. Tick marks on the x-axis indicate 10 Hz intervals.


5 Hz
20 Hz
150 Hz
Threshold (µm)
60
45
30
15
0
0
20
40
60
80
100
120
140
Frequency (Hz)

responses had CoVs that were more than the 0.30 cutoff for SA II but less than the 0.50 cut off for SA I responses. The Chubbuck stimulator was not used on a comparable number of Nile crocodile afferents, but based on classification of 15 afferents from 2 Nile crocodiles using handheld probes, similar proportions of RA and SA units were found (SA 55% and RA responses 45%).

Among a set of RA responses (n=14), neurons were maximally phase-locked with one-to-one correspondence of response per stimuli cycle to the lower vibrations (10 to 35 Hz) and were less attuned to 100 Hz, 200 Hz, 300 Hz, and higher frequency stimuli (Fig. 8). Furthermore, smaller displacements of the probe were required to elicit responses for 20-30 Hz vibrations compared to lower (5 and 10 Hz) or higher (50, 75, 100, 150, 200 Hz) frequency stimuli. RA units continued to respond to frequencies greater than 350 Hz in 4 cases, and the median highest frequency for the SA responses was 250 Hz in the SA II units.

In order to test for other possible sensory functions of the cranial ISOs, we monitored activity in response to hyperosmotic solutions and to electric fields (n= 40 afferents in 4 alligators and n=15 afferents in 2 crocodiles). Single unit neuronal responses were isolated as described above, and cranial regions were exposed to room temperature deionized water and 31 to 47 ppT sea salt solutions. These were applied via pipette or swab to the specific receptive field and allowed to remain for at least 3 minutes. No single or multiunit activity was detected apart from responses to the force of the initial application of the solution. In other cases, the head of the crocodilian was lowered into a tank of room temperature water, immersing the previously identified receptive field

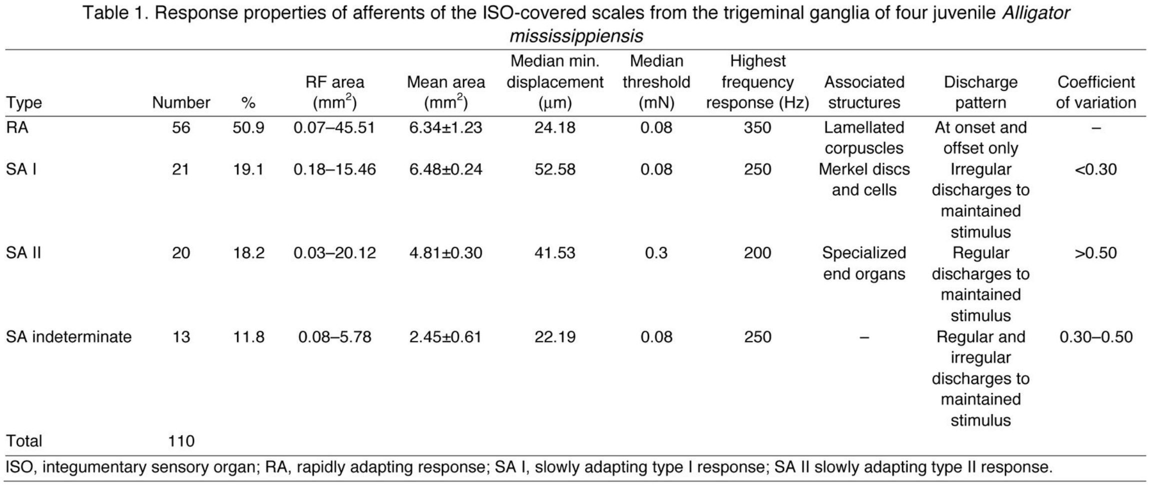


Table 1. Response properties of afferents of the ISO-covered scales from the trigeminal ganglia of four juvenile *Alligator mississippiensis*

Type	Number	%	RF area (mm^2)	Mean area (mm^2)	Median min. displacement (μm)	Median threshold (mN)	Highest frequency response (Hz)	Associated structures	Discharge pattern	Coefficient of variation
RA	56	50.9	0.07–45.51	6.34±1.23	24.18	0.08	350	Lamellated corpuscles	At onset and offset only	–
SA I	21	19.1	0.18–15.46	6.48±0.24	52.58	0.08	250	Merkel discs and cells	Irregular discharges to maintained stimulus	<0.30
SA II	20	18.2	0.03–20.12	4.81±0.30	41.53	0.3	200	Specialized end organs	Regular discharges to maintained stimulus	>0.50
SA indeterminate	13	11.8	0.08–5.78	2.45±0.61	22.19	0.08	250	–	Regular and irregular discharges to maintained stimulus	0.30–0.50
Total	110									

ISO, integumentary sensory organ; RA, rapidly adapting response; SA I, slowly adapting type I response; SA II slowly adapting type II response.

Table 1. Response properties of afferents of the ISO-covered scales from the trigeminal ganglia of 4 juvenile *Alligator*.

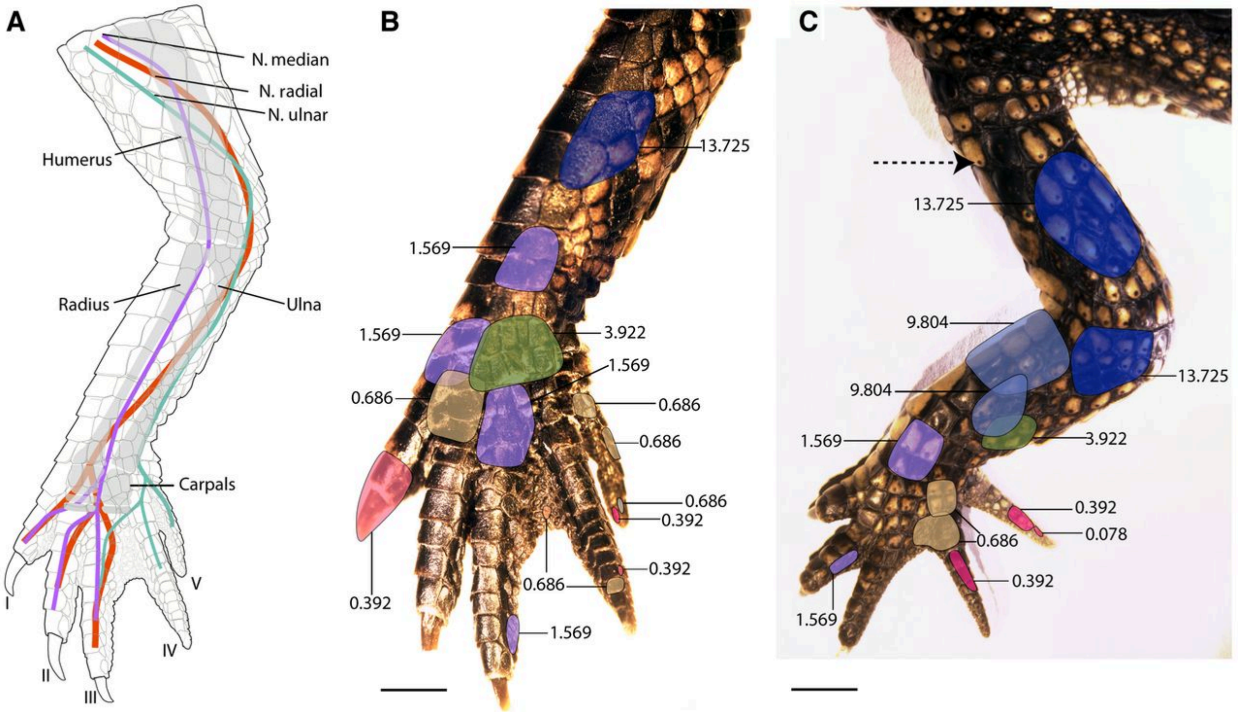
while the electrode was held in place. A 9V battery was placed in the water and moved in different configurations around the head, similar to paradigms used to elicit electrolocating behaviors in platypus (Scheich et al., 1986). No single or multiunit responses were observed.

Responses of the spinal nerves

The forelimb of the crocodile was supplied by the median, radial, and ulnar nerves, and all three ran to the 5 digits as well as to the skin of the dorsal surface of the limb (Fig. 9A). The three nerves were exposed near the proximal humerus. An electrode was inserted into the nerve, and single unit responses were recorded. In total, 67 single units from 2 alligators, and 45 units from 2 crocodiles were examined from the medial, radial, and ulnar nerves. For both body regions, receptive fields were drawn on the photographs of the animal.

The receptive fields found on the limbs of the alligators ranged from less than 1 mm^2 to more than 58 mm^2 (average = 16.4, SD= 11.5), and the largest were found on the anterior surface of the hindlimb, above the tibia. On the forelimb, the smallest examples were isolated to the distal regions of the digits of the forelimb. In particular, digits 4 and 5 had minute receptive fields near the “fingertip” areas. These digits are notable in that they lack the claws found on digits 1 to 3, were more slender, and appeared to be proportionally reduced in crocodilians compared to other reptile groups (Vargas et al., 2008). They have also been speculated to have a specialized tactile role in detecting

Fig. 9. Receptive fields of the forelimb in crocodilians. **A.** In order to record from the forelimb, individual single units were isolated in the median, radial, and ulnar nerves near the proximal areas of the humerus. **B.** Select receptive fields of single peripheral afferents from an *Alligator* case. The numbers represent the indentation threshold from the von Frey filament, measured in millinewtons. Scale bar is 1 cm. **C.** Select receptive fields in a *C. niloticus* case. Individual body ISOs are visible as small black dots on each scale (black dotted arrow). Same conventions as in (B).


A
N. median
N. radial
N. ulnar
Humerus
Radius
Ulna
Carpals
I
II
III
IV
V
B
13.725
1.569
1.569
3.922
1.569
0.686
0.686
0.686
0.686
0.392
0.392
0.686
0.392
1.569
C
13.725
9.804
13.725
9.804
1.569
3.922
0.392
0.686
0.078
0.392
1.569

tactile stimuli from aquatic prey (Vliet and Groves, 2010). Numerous low threshold receptive fields were found distributed on the distal portion of these digits as well, with afferents responding to indentation forces of 0.392 mN or less. There was an orderly progression of sensitivity as one moved more proximally up the limb with the dorsal surface of most digits responding to the forces between 0.686 and 1.569 mN, to regions covering the carpals responding to 0.392 to 9.804 mN, then to areas covering the radius and ulna responding to forces of 13.725 mN (Fig. 9B,C). Other areas of heightened sensitivity included the webbing between digits 1 through 3.

The hindlimb followed a similar pattern such that afferents from distal portions of the digits had small receptive fields and responded at the lowest thresholds, whereas afferents innervating areas over the tibia and fibula had larger receptive fields and responded at higher thresholds. By exposing the median and saphenous nerves near the proximal end of the femur, recordings were obtained for much of the hindlimb and its plantar surface (Fig. 10). In recordings from the fore- and hindlimbs in both species of crocodilians, both RA (n=18 in Nile crocodiles; n=29 in alligators) and SA afferents (n= 27 in Nile crocodiles; n=39 in alligators) were observed.

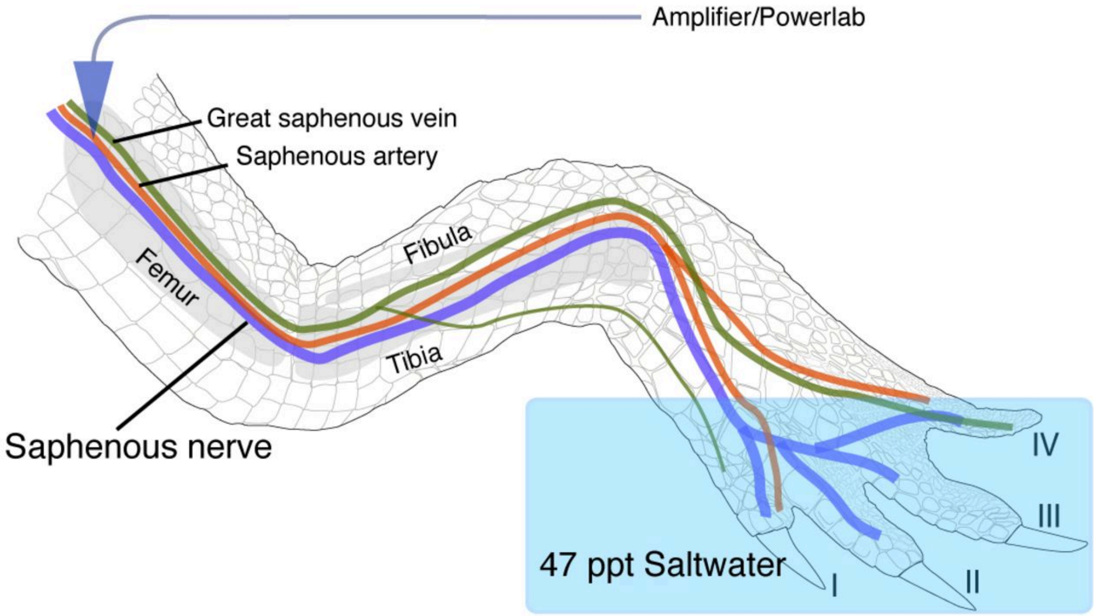
In all of the electrophysiological recordings, the relationship between receptive field surface area and the threshold force necessary to elicit activity was noted. In both alligators and Nile crocodiles, on the face (Spearman's $\rho = 0.651$; n= 127, $p < 0.001$ in alligators and Spearman's $\rho = 0.5664$; n= 16, $p = 0.0222$ in crocodiles) and body (Spearman's $\rho = 0.618$; n= 67, $p < 0.001$ fields in alligators and Spearman's $\rho = 0.6506$; n= 45, $p < 0.001$ in crocodiles), smaller receptive fields were correlated with lower displacement forces. Likewise, larger receptive fields were correlated with greater displacement forces.

We also tested for responses to salinity changes or electric fields. The receptive field of interest on the limb was identified and examined for threshold sensitivity and then the animal was positioned to allow the body region to be submerged in a container filled with distilled water or 31 to 47 ppT sea salt solution (Fig. 10). Immersing the limb in room temperature water or hyperosmotic sea salt solution, as monitored for at least 3 minutes, evoked no observable single or multiunit responses in either alligators or crocodiles. Similarly, there were no responses to the 9V battery in the water.

Behavior

Juvenile crocodilians, ranging in age from hatchling (SVL=10.2 cm) to 3 years (SVL=76.2 cm) were observed and videotaped orienting towards and capturing prey or ingesting food pellets dropped into the water. With full spectrum lighting, they generally turned rapidly towards water ripples generated by dried food pellets dropped from above. Often, they closed both their lower, movable nictitating membrane and external eyelids as they snapped laterally towards the initial source of the disturbance. Although the jaws often secured the food with the initial bite, subsequent bites re-orienting toward the item appeared to rely on direct contact with the submerged pellet since the closed eyes were positioned well above the water surface (Movie 1, clip 1). These sideways snaps of the

Fig. 10. Electrophysiological recording preparation as used in body recordings. In this case, the single unit responses from the hindlimb were recorded from the saphenous nerve. Once units were identified mechanically, the receptive field was submerged in hyperosmotic solutions to monitor for activity. No activity related to immersion in the seawater solution was detected.

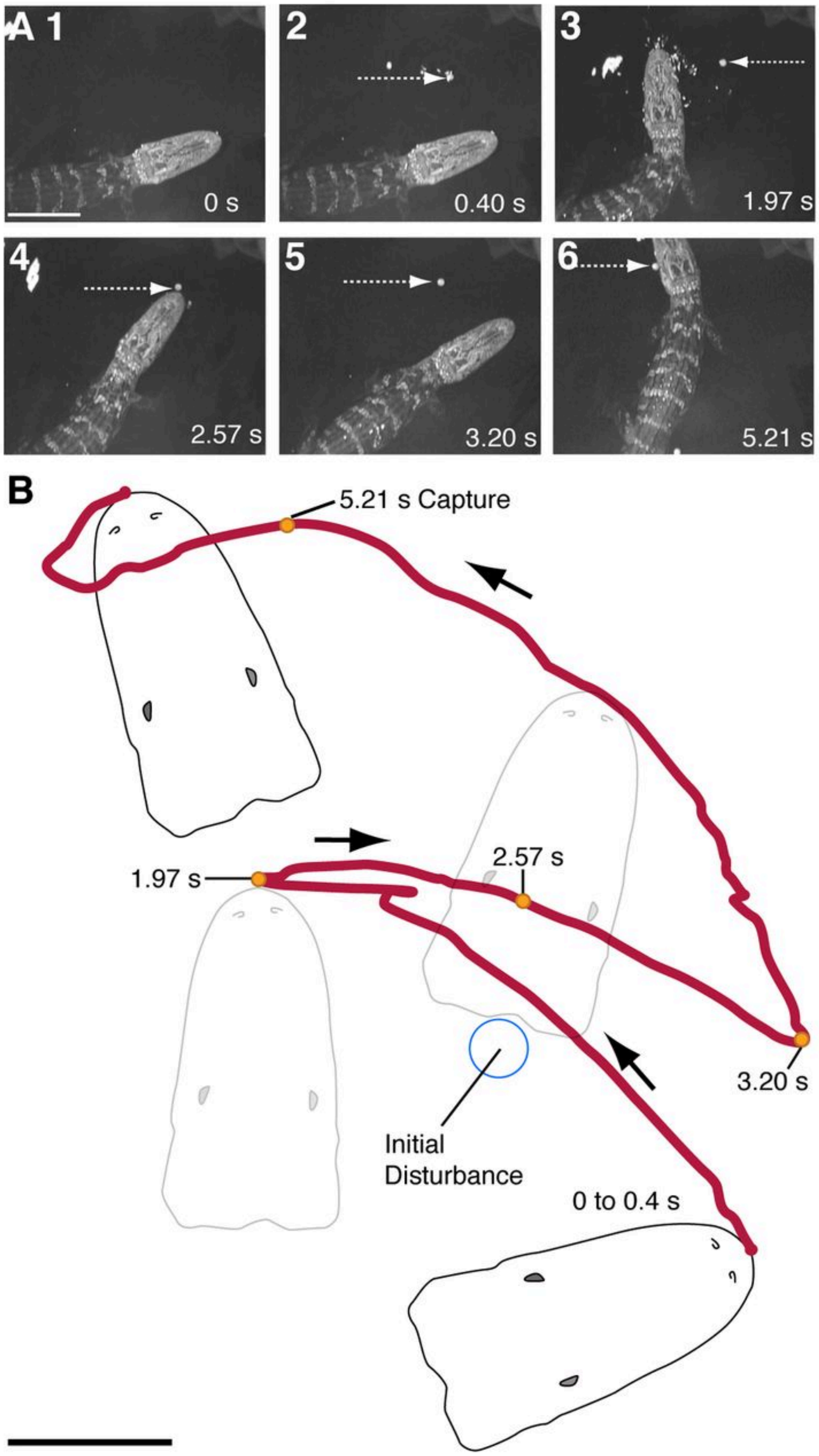

Amplifier/Powerlab
Great saphenous vein
Saphenous artery
Fibula
Femur
Tibia
Saphenous nerve
IV
III
47 ppt Saltwater
I
II

jaws were directed toward the food pellet within 50 to 70 msec of the item's contact with the skin.

Crocodilians were also monitored under 940 nm IR illumination and with white noise to block auditory cues. Both alligators and crocodiles were capable of orienting towards the location of water disturbances when floating with their heads at the water surface. When positioned in this manner, the areas of greatest ISO density near the rostral margins of the jaws and adjacent to the teeth were often below the air-water interface and just the eyes, ear flap, and more dorsal regions of the maxilla were exposed. Following the initial directed movement towards the water disturbance (Movie 1, clip 2), both alligators and crocodiles often swept their heads laterally when in the area of the source of the ripples (Movie 1, clips 3-4 and Figure 11). The animal was obviously searching for the source of the disturbance and often continued for 3-4 seconds or until its jaws touched an object. Within 200 msec of contact with the object, the crocodilian usually bit the item and began to attempt ingestion. In the event that the animal had inadvertently secured a non-edible item (such as floating aquarium fauna), the object was released after several snaps of the jaws whereas edible objects were quickly eaten.

Crocodilians were also observed orienting towards freely swimming fish under IR illumination. Despite facing the opposite direction and having their heads above water, crocodiles were capable of rapidly turning and diving underwater towards the location of the fish (Movie 1, clip 5). In another predatory strategy, crocodiles would often remain submerged until prey came into contact with the skin surfaces (Movie 1, clip 6) or the open mouth (Movie 1, clip 7), at which point the animal immediately attempted to capture the fish in its jaws.

Fig. 11. Crocodilian behavioral responses following water surface disturbance. **A.** Individual images from a film sequence recorded under infrared lighting and white noise presentation to block audition as a juvenile *Alligator* orients towards a surface wave generated by a small food pellet (white arrow). **B.** Schematic of the orienting movements presented in (A). From the animal's initial location, a lateral, sweeping head movement is repeated until the head makes tactile contact with the floating pellet and rapidly captured. Scale bar is 10 cm.


A 1
0 s
2
0.40 s
3
1.97 s
4
2.57 s
5
3.20 s
6
5.21 s
B
5.21 s Capture
1.97 s
2.57 s
3.20 s
Initial Disturbance
0 to 0.4 s

Discussion

Alligatorids have a dense array of sensory receptors (integumentary sensory organs –ISOs) extending around the mouth and cranial regions (4200 ISOs, S.D.=94 in *A. mississippiensis*) whereas *Crocodylids* have ISOs distributed across almost every scale of the body surface (6200 ISOs, S.D.=389) as well as on the head (2900 ISOs, S.D.= 134 in *C. niloticus*). Since the earliest reports of ISOs (Maurer, 1895; von Wettstein, 1937) and their use in the dichotomous identification of crocodilian skins (King and Brazaitis, 1971), their function has remained a topic of speculation. Although detailed morphological studies undertaken in *Caiman* receptors (von Düring, 1973; von Düring, 1974; von Düring and Miller, 1979) strongly suggested a mechanosensory role for ISOs, physiological characterization of their function has been limited to the trigeminal receptors of a single species (*A. mississippiensis*) (Soares, 2002). Anatomical studies of crocodylid post-cranial ISOs from *C. porosus* focused on a potential role of the organs as osmoreceptors (Jackson and Brooks, 2007; Jackson et al., 1996). This hypothesis is based in part on models of how ISOs mechanically flatten under osmotic pressure in a saltwater environment and on experiments measuring the mass of water consumed by the estuarine crocodiles. This led to the hypothesis that ISOs are the first identified vertebrate integumentary osmoreceptors (Jackson and Brooks, 2007). Other investigators have proposed that ISOs could function as magnetoreceptors (Rodda, 1984) or electroreceptors (Bullock, 1999).

Structure of Integumentary Sensory Organs

The ISOs appear to share many structural similarities with known mechanoreceptors. These include the push-rod receptor organs distributed across the snouts of monotremes (Andres and von Düring, 1984; Andres et al., 1991) and the Eimer's organs found on the glabrous skin on the rhinarium of moles. (Catania, 1995). Numerous "tastflecke" found on the small warts of bufonid toads and ranid species of frogs have also been identified (Lindblom, 1963; Ogawa et al., 1981). Cutaneous cephalic corpuscles with protruding centers appear in some Colubrid snakes (Jackson, 1971; Jackson and Doetsch, 1977). Herbst and Grandry corpuscles comprise the tactile bill tip organs found in ducks (Berkhoudt, 1979; Gottschaldt and Lausmann, 1974). In all these cases, the receptor appears as smooth, domed structure with an apex suitable to transducing deflection to a series of specialized afferents.

In juvenile crocodilian ISOs, the external, keratinized dome typically had a diameter of 0.5 mm or less for those distributed across the jaws whereas larger ISOs (1.2 mm) were found on crocodylid body scales. Despite this size difference, both populations of ISOs appeared remarkably similar in internal composition. The stratum corneum is thin over the organ (5 μm), presumably allowing a range of motions to compress the structure. This layer of 3 to 5 β-keratin cells (Alibardi, 2010) functions both in structural integrity of the ISO and acts as scaffolding for the most apical of the fine nerve terminals. In transverse sections, highly-branched melanocytes can be seen throughout the keratinized layers and underlying collagenous layers and impart the distinctive pigmentation seen in most of the ISO bodies. A number of mechanoreceptors are apparent in sectioned ISOs. These mechanoreceptors can be broadly categorized based on their morphology and distribution, as described by von Düring and Miller (1979). These distinctions are as follows: 1) receptors of the epidermis, 2) receptors of the connective tissue with Schwann cell elaborations or myelination, 3) receptors of the connective tissue lacking Schwann cells, and 4) Merkel cell neurite complexes. Among tactile specializations of the first group, crocodilians, as well as reptiles more generally (Landmann and Villiger, 1975; von Düring, 1973), are notable for having expansions of the receptor terminals, compared to the finer, tapered free nerve terminals found in most other vertebrates (Fig. 4B). The dermal Merkel column, similar to the ubiquitous epidermal Merkel neurite complex, traditionally has been interpreted as slowly-adapting in other species. These columns were isolated to regions under each ISO whereas similar Merkel cells are found ubiquitously across the epidermal body surface in fishes (Lane and Whitear, 1977), amphibians (Nafstad and Baker, 1973), birds (Nafstad, 1971), and mammals (Halata, 1970; Munger, 1965). Lamellated corpuscles, comparable to the Paciniform structures of mammals (Pease and Quilliam, 1957) have been characterized as rapidly-adapting (Andres and von Düring, 1973; Iggo and Muir, 1969; von Düring and Miller, 1979). Indeed, both rapidly adapting and slowly-adapting afferents were observed in our physiological data.

The close association between the discoid terminals and the supporting epidermal cells of the stratum corneum and lucidum has been observed before in reptile scales (von Düring and Miller, 1979) and in mammalian glabrous skin (Munger and Ide, 1988), and this relationship also holds for the crocodilian ISOs both from the cephalic and body regions. Highlighting the intimate association with the free nerve terminals, fluorescent lipophilic label (DiI) applied to bundles of myelinated fibers of the maxillary nerve often labeled the keratinized epidermal layers directly over the ISO while remaining absent from adjacent scaled regions.

Several features of the trigeminal system of crocodilians stood out when examining the innervation of the cranium. First, there was an exceptional density of nerve fibers supplying the skin of the face and a vast network of branching nerve bundles just below the epidermis. Throughout the dermis, ensheathed groups of myelinated afferents projected across the rostro-caudal length and outwards towards the epidermis, as seen in the cleared Sudan Black B specimens. The bundles emerged through small foramina of the maxilla and dentary. This organization is reminiscent of mechanosensory end organs found in the foramina of anterior margins of the beaks of water-foraging birds with bill tip organs (Cunningham et al., 2010) and highlights the shared archosaurian phylogeny between crocodilians and birds (Hedges and Poling, 1999). It seems likely that by having the majority of the maxillary and mandibular nerves shielded in bone, crocodilians are armored against many potential injuries as might be encountered when feeding communally while simultaneously maintaining an acutely sensitive skin surface via the fibers running through the foramina.

Trigeminal Afferents and Their Organization

A large proportion of the neurons in the trigeminal ganglion responded to stimulation of the areas most densely covered in ISOs near rostral points of the pre-maxilla and mandible and surrounding the teeth. In addition, many afferents responded to very light contact to the teeth, underscoring previous ultrastructural investigations of sensory nerve endings within the dental ligament and attachment tissues in *Caiman* *crocodilus* (Berkovitz and Sloan, 1979; Tadokoro et al., 1998). In general, the smallest receptive fields were found rostrally on the upper and lower jaws and near the teeth. This overall pattern of small receptive field size and corresponding "overrepresentation" in the ganglion is reminiscent of cortical magnification of behaviorally important skin surfaces observed in mammals (Krubitzer, 2007; Sur et al., 1980). For many species, the most important skin surfaces used for exploring objects are densely innervated by afferents with the smallest receptive fields, and the skin surfaces have correspondingly large representations in the central nervous system. Examples of functionally significant skin surfaces with consequently large nervous system representations include the forelimb of the raccoon (Welker and Seidenstein, 1959), the bill surface in the platypus(Pettigrew, 1999), and the lips and tongue of humans (Penfield and Boldrey, 1937). The overall pattern found for crocodilians, which have the highest density of ISOs and smallest receptive fields around the teeth, provides an important clue to ISO function. We suggest ISOs play a key role not only in capturing prey based on water movements (Soares, 2002) and contact but also in discriminating objects that have been grasped in the jaws and guiding the manipulation of prey once it has been secured. This interpretation is consistent with other recent findings in vertebrates that have revealed very large cortical representations of the dentition and oral structures that had been previously unappreciated(Jain et al., 2001; Kaas et al., 2006; Remple et al., 2003)

Within the ganglion, neurons that responded to the rostral head were typically located ventrolaterally whereas neurons responding to stimulation of the caudal regions of the jaws were positioned dorsomedially. As would be expected, responses to stimulation of the pre-maxilla, maxilla, and quadratojugal of the upper jaw were recorded from the anterior regions of the ganglion, in proximity to the entrance of the maxillary nerve into the ganglion, and areas responsive to stimulation of the dentary were recorded from the posterior regions, near the mandibular nerve's division from the ganglion. The electrophysiologically-derived topography of the crocodilian trigeminal ganglion was consistent with maxillary representations in the maxillo-mandibular lobe as documented in HRP tracer studies from hatchling chicks (Noden, 1980).

In both the Nile crocodiles and alligators, receptive fields, some as small as the area of a single ISO, were sensitive to indentation thresholds produced by the finest von Frey filaments corresponding to a force of 0.078 mN. These measurements represent sensitivities more acute than those of primate fingertips (Johansson et al., 1980) – skin surfaces that are widely appreciated for their sensitivity (Darrian-Smith, 1984; Kaas, 2004). Similarly, tactile responses were elicited by mechanical displacements as small as 3.9µm - an indentation threshold lower than found for the human hand (Johansson, 1978). These findings are evidence of the extreme and surprising sensitivity of the crocodilian face and may represent a requirement for the detection of subtle water disturbances (Soares, 2002) in addition to the discrimination of different objects and prey.

RA, SA I, and SA II type responses were identified in recordings from the surface of single trigeminal ISOs of alligators as well, in keeping with the diverse array of mechanoreceptors and afferent end organs found in each receptor. Prior electrophysiological studies from the plantar nerve of alligators and caiman, which lack ISOs on the body, have also found RA, SA I, and SA II afferents on the hindlimbs (Kenton et al., 1971). However, in these preceding studies, the finest indentation forces found for these cutaneous regions lacking ISOs (as is the case in alligatorid limbs) were more than six times greater than the median indentation forces for responses from the ISO covered areas (0.08 mN) in this report, suggesting that the organs provide a considerable increase in sensitivity. ISOs therefore seem to be a structure that imparts great sensitivity to an otherwise armored and shielded body surface.

Analysis of 14 RA responses collected for stimulus frequencies up to 350 Hz indicated that the lowest indentation thresholds were found at 20 to 30 Hz within the 5 to 150 Hz range. Larger displacement distances were necessary to elicit 1:1 entrained responses of the afferent to frequencies both below and above the 20-30 Hz window. The 20 Hz vibration stimulus has been noted as one of the optimal frequencies to induce orientation behaviors towards water surface disturbance in *Notonecta glauca* – a predatory aquatic insect that localizes and orients towards prey-borne surface waves transmitted via mechanoreceptive tarsal scolopoidal organs and abdominal sensory hairs (Lang, 1980; Weise, 1974). Thus the tuning of afferents to this frequency in crocodilians is consistent with prior behavioral observations of juvenile alligators orienting towards water surface ripples (Soares, 2002). In addition, responses from SA (both types I and II) and RA units often extended beyond 200 Hz and 300 Hz and were elicited by 40 to 80 μm displacements, suggesting that relatively higher frequency vibrations can also be readily transduced by ISOs.

Spinal Nerve Afferents

As one of the goals of this project was to collect physiological data regarding sensory function of post-cranial ISOs in crocodiles, it was necessary to record from spinal nerves innervating the integumentary surface. Although still responding to forces of

13.725 mN and finer, afferents from the body were not as sensitive as those distributed across cephalic regions in either crocodiles or alligators. Discrete single units across the limbs were typically large except for those found in distal regions of certain digits (IV and V on the forelimb and IV on the hindlimb). There also appeared to be sensitive regions, responding to indentation forces of 0.392 and 0.686 mN, on the webbing present between digits III and IV on the forelimb. These results are consistent with the concept of the ISOs being discrete tactile receptor units as they are present on some of the smallest scales of the body; perhaps the increased receptor density per unit area imparts a greater degree of acuity. This idea is supported by the notion that the digits IV and V of the forelimb, which are notably more slender and do not have the claws found on the other digits, might be adapted to detecting somatosensory cues when the animal is floating in the water (Vliet and Groves, 2010). When foraging for fish, *Caiman yacare* partially open their mouths and fully extend their forelimbs, adopting a “cross posture,” and indeed, fish have been observed nipping at the caiman’s digits (Olmos and Sazima, 1990) suggesting that tactile information from the digits could mediate predatory behaviors.

Another motivation for physiological investigation of the integument comes from Jackson’s intriguing series of experiments into the potential osmoreceptive capabilities of post-cranial ISOs of crocodiles (Jackson and Brooks, 2007; Jackson et al., 1996). However, in recording directly from the afferents innervating ISO-covered body surfaces in Nile crocodiles, no single or multiunit responses attributable to exposure to hyperosmotic solutions were observed. The results were similar to those from alligators which were used as an experimental control without body ISOs. Finally, no responses were detected in response to electrical stimuli (Scheich et al., 1986), suggesting ISOs play no role in electric field detection, and by extension, that crocodilians do not have electroreception.

We suggest the crocodilian ISOs function as part of an elaborate mechanosensory system and are adaptive to a number of aquatic behaviors. When filmed under 940 nm IR illumination, both crocodiles and alligators readily struck at and captured fish (Movie 1, clip 1) and occasionally oriented towards minute water surface disturbances, similar to the results reported by Soares (2002). Beyond providing positional cues to the source of the stimuli, the ISOs are densely distributed throughout the upper palate and areas adjacent to the teeth within the oral cavity – a location unlikely to receive and transduce the pressure from expanding surface waves. Disjunct regions of greater receptor density were observed near the eye and nares - similar to supraorbital and rhinal microvibrissae areas as found in mammals relying on trigeminally-mediated tactile discrimination (Brecht, 2007; Ling, 1966; Lyne, 1959). When actively foraging, crocodilians open their mouths and move so as to sweep the arrays of cranial ISOs across the surface and underwater, rapidly capturing and securing objects that make contact with their heads, and releasing any non-edible matter, indicating that it is likely that they can discriminate between multiple different materials using tactile cues alone. As a testament to these discriminatory abilities, mother crocodilians often manipulate their eggs as they begin hatching, gently cracking away the shell with their teeth (part of a feeding apparatus capable of inflicting crushing bites and dismembering large prey) and allowing the hatchlings to seek protection in her mouth (Hunt, 1987; Pooley and Gans, 1976) – a situation in which blunted tactile acuity would be maladaptive. Although the question remains as to why ISO distribution differs between the alligatorid and crocodylid species, results from recording from the spinal nerves suggest that both species tested are sensitive to low thresholds of force. Some have speculated that ISOs homologous to the post-cranial populations of crocodylids are present far deeper within the integument in alligatorids (Richardson et al., 2002). While crocodilians are certainly capable of accurately ambushing and capturing prey by relying on their acute visual systems (Heric and Kruger, 1966; Pritz, 1975) in lighted conditions, even on the darkest nights, prey still face a formidable mechanosensory system if they unexpectedly come into contact with these reptiles.

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CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

Through examinations of the sensory systems of diverse groups of vertebrates, much can be appreciated about the historical pressures that have shaped unique behavioral traits and, consequently, the organizational changes that have arisen within their nervous systems. These issues are particularly intriguing when observing a range of animals that have either become specialized to a particular habitat or prey (i.e., the completely aquatic tentacled snakes and their piscivorous diets) to those species that have a more generalist approach to both environment and prey selection, as in the case of many crocodilians and the water shrews. Sensory biology as viewed through the lens of neurobiology is well-suited to shed light on many aspects of animal behavior and its neuronal mechanisms (Thewissen and Nummela, 2008). These advantages arise from the field's position at the junction of physiology, anatomy, systematics, and molecular biology, to name a few, as well as the necessity of having a familiarity with an organism's typical behaviors to place the sensory percepts in some context. It is with these multidisciplinary questions in mind that these studies were undertaken in the hope that both general trends in the relationship between behavior and its neural circuitry can be elucidated, as well as the properties of some unique vertebrates. These have been conducted largely in the somatosensory system; however, further studies examining the functional and evolutionary relationships of behavior in other sensory modalities are likely to be a source of more interesting questions.

Insights on the Insectivores and Water Shrews

In line with some of the well-understood mammalian models in neurobiology, the Eulipotyphlan "insectivores" provide a unique perspective on the possible organization of the brains of stem mammals (Gebo, 2004; Kielan-Jaworowska et al., 2004). While some of the phylogenetic relationships between members such as the talpid moles and soricine shrews are under contention (Grenyer and Purvis, 2003; Mouchaty et al., 2000; Symonds, 2005), the results as presented in Chapter II underscore the sister group relationship between the two groups. Strikingly similar arrangements of the most basic cortical areas (primary somatosensory, visual, and auditory cortex) are found adjacent to one another in caudal cortex, with little area left in between. A distinct rhinal sulcus divides the smooth neocortex from the lateral piriform cortex.

Like many shrews, the American water shrew (*Sorex palustris*) has a distinctive set of whiskers distributed across the front of the face and near the nostrils. Neocortical representations from these prominent vibrissae occupy a large area of both primary somatosensory (S1) and second somatosensory (S2) cortex, in a configuration reminiscent of the expansion of S1 and S2 as observed in the star-nosed mole (Catania and Kaas, 1995). These somatotopic maps are located in S1's inverted homunculus against a mirror-image representation in S2. Although the much-appreciated cortical barrel representations for each individual whisker are unclear, preliminary evidence indicates that prominent whisker representations are present in nuclei of the trigeminal brainstem of the water shrew (Catania, unpublished observations). A series of anatomical investigations to identify sub-cortical whisker representations seems worthwhile to evaluate the water shrew's impressive tactile periphery in light of more widely-studied rodent models.

Another facet of water shrew biology that merits closer examination is their unique repertoire of predatory behaviors. Although several studies have clarified the use of movement and tactile cues in guiding striking behaviors in the water (Catania, 2013; Catania et al., 2008) and even uncovered the unconventional methods they employ to sample odorants underwater (Catania, 2006), it would be interesting to see these similar results interpreted alongside other shrew species. With a greater understanding of the composition of their habitats and their nervous system architecture, some previously-noted abilities seem suspect, such as their ability to make use of ultrasonic vocalizations as a form of echolocation (Gould et al., 1964 ; Siemers et al., 2009); however, these reevaluations are not meant to present shrews as primitive, unsophisticated animals. As some of the smallest extant mammals and given the suggestion that ancestral mammals were similarly small-bodied, shrews provide a unique opportunity to study the limits of how diminutive a nervous system can become yet continue to produce effective behaviors (More et al., 2010). Whether there are adaptations to the peripheral nervous system that affect conduction velocity or changes to the central nervous system that refine sensory system processing with a limited neuronal substrate is currently unknown. These questions could be answered by examining a range of insectivores of different body sizes including the masked shrew (*S. cinereus*) with an average total mass of 2 to 5 g or the

Etruscan pygmy shrew (*Suncus etruscus*) which at 1.6 g in body mass is the smallest extant mammal and the subject of several recent neurobehavioral investigations (Naumann et al., 2012; Roth-Alpermann et al., 2010).

Insights on Tentacled Snakes

Converging lines of anatomical, physiological, and behavioral data suggest that the rostral appendages of the tentacled snakes (*Erpeton tentaculatus*) function in mechanoreception. No evidence was found for their use in fish luring or electroreception.

Following the physiological work reported in Chapter III, Catania demonstrated that newly born tentacled snakes are capable of adopting the J-shaped hunting posture upon their first exposure to live fish (Catania, 2010). When separated from live fish by a translucent barrier, these snakes aimed and struck at the anticipated location of a fish making a reflexive C-start escape movement. These results suggest that the juvenile snakes are not newly acquiring their predatory behaviors from prior success capturing prey but are innately exploiting the fishes' escapes to their advantage. Moreover, these observations underscore the role of visual cues in directing the tentacled snakes' behavior – a finding that is congruent with the electrophysiological work from the snake's optic tectum. The superficial layers of the tectum had robust single unit responses corresponding to the contralateral visual fields, and deeper layers contained neurons responsive to tactile stimulation of the body, face, and tentacles. These retinotopic and somatotopic orientations were largely in register with one another, with caudal areas of the body represented in posterior areas of tectum whereas the expanded representation of the snake's head was found towards the rostral portions. As a structure with well-established roles in integrating different sensory modalities and directing movement (Stein and Meredith, 1993), the optic tectum (the homolog of the mammalian superior colliculus) is of particular interest in vertebrates that appear to make highly coordinated movements in response to sensory stimuli. This is also the case in pit vipers (Hartline et al., 1978) and boids (de Cock Buning, 1983) which are able to synthesize visual and infrared-related information to perform accurate strikes towards their endothermic prey. Examining the tectal organization of other reptiles that make ballistic movements to capture prey, such as Mata mata (*Chelus fimbriata*) or alligator snapping turtles (*Macrochelys temminckii*), might prove fruitful in providing further neural correlates of unusual behaviors mediated by tactile or visual systems.

From a broader perspective, the evolution of the tentacled snakes' appendages is interesting to consider. While other taxa of snakes such as the sea snakes (genus Hydrophiinae) are highly adapted to aquatic habitats (Brischoux and Shine, 2011), the tentacled snake is the sole member of its genus Erpeton within the Homolopsid order. Another homolopsid snake, the dog-faced water snake (*Cerebrus rynchops*) shows swellings at the rostral labial regions where the appendages are found on *Erpeton* (Winokur, 1977), and one can imagine a selective advantage in having highly-sensitive regions on the face with these growing more elaborate over time. It is plausible that these scaled mechanosensory specializations could eventually result in an analog of the rodent whiskers or the distinctive rays of the star-nosed mole.

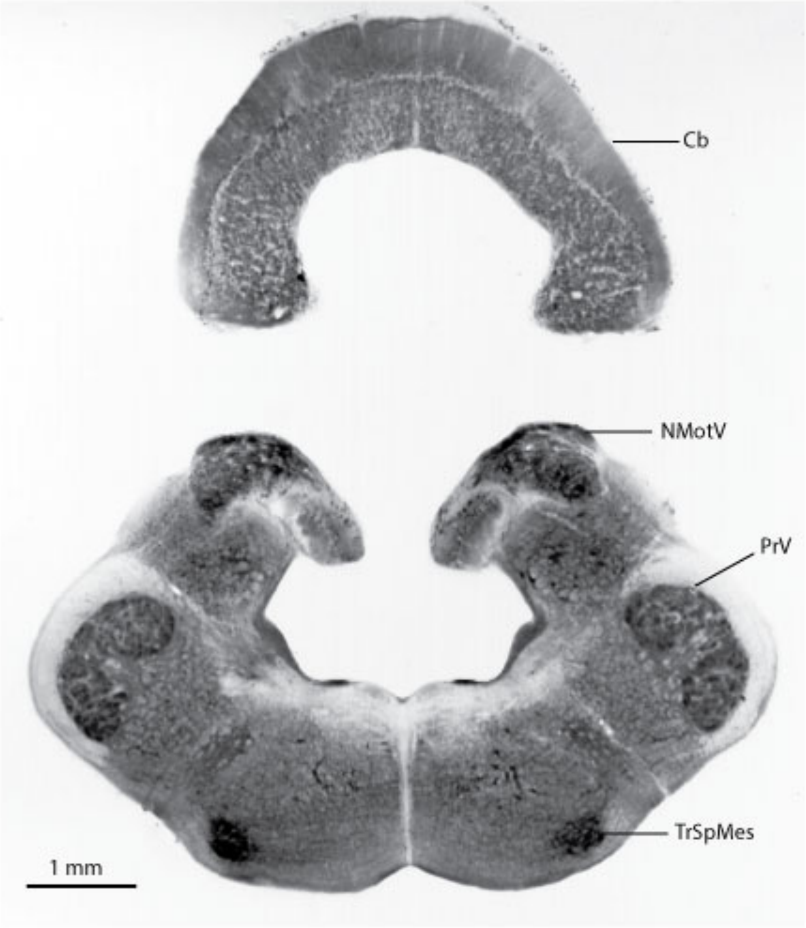
Insights on Crocodilians

With the goal of examining the integumentary sensory organs (ISOs) in a ethological context, we have observed that the ubiquitous receptors seem to be highly-sensitive mechanoreceptors and appear capable of mediating a number of crocodilian behaviors for which an acute tactile system would be advantageous. Along with their famed predatory abilities, crocodilians display an unusually great range of social behaviors among reptiles (Garrick and Lang, 1977; Vliet, 1989). One example is the maternal care that female crocodilians provide for their offspring which is manifested in the form of aggressive behaviors that could reduce infanticide, assistance in manipulating the hatchling out of their shells using their teeth, and allowing the hatchlings to seek refuge inside of the mother's mouth (Hunt and Watanabe, 1982; Pooley and Gans, 1976). In each of these situations, a sophisticated sense of touch would be beneficial (and indeed, a poor sense of touch could be disastrous in the case of the hatchlings hiding within the mouth). The notion of a sensitive tactile sensory surface seems counterintuitive to heavily-armored, osteoderm covered bodies of the crocodilians (Vickaryous and Hall, 2008) which are capable of engaging in forceful, potentially physically-damaging actions when attempting to overpower large mammalian prey. However, it appears that the ISOs have evolved as a solution to this dilemma. Numerous mechanoreceptive afferent specializations (e.g., Merkel cells, lamellated corpuscles, free nerve endings) are concentrated in behaviorally significant locations of the face and body, and the innervating nerves run through layers of thick bone to emerge through foramina proximally to small groups of ISOs. This could afford a degree of protection of the nerves while still providing a mechanism for exquisite touch or pressure sensitivity.

In creating a more complete picture of the function of the ISOs relative to crocodilian behaviors, there are multiple realms that seem worthy of further investigation. One basic question is the distribution of the ISOs in other species, beyond the American alligator and Nile crocodile. Preliminary observations from the African slender-snouted crocodile (*C. cataphractus*) and Siamese crocodile (*C. siamensis*) suggest that they have similar total numbers and densities of ISOs, but it would worthwhile to expand these analyses to other morphologically distinctive species such as gharials *(Gavialis gangeticus*) especially considering their fish-specific diets (Thorbjarnarson, 1990).

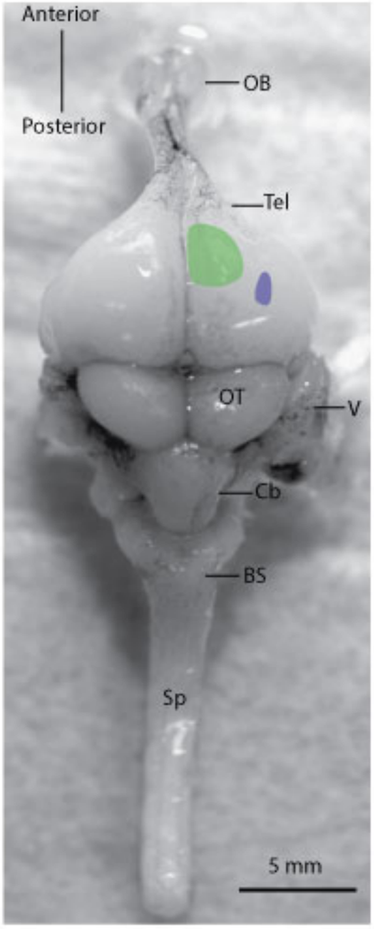
Developing from the electrophysiological recordings from the trigeminal ganglia and peripheral nerves, future investigations of sensory (particularly mechanosensory) representations in other areas of the central nervous system seem warranted. These could include the trigeminal nuclei of the brainstem (Fig. 1) which appears as a large elliptical structure at the ventrolateral edges of the coronal sections (Huber and Crosby, 1926). It is probable that there are representations of the body within deeper layers of the optic tectum and torus semicircularis, as suggested by axonal degeneration studies from the dorsal column nuclei (Pritz, 2002; Pritz and Stritzel, 1989). In a topographic configuration unique to birds, areas of the body innervated by the trigeminal nerve (i.e., the head) are represented in a discrete region of the telencephalon called the nucleus basalis (Berkhoudt et al., 1981; Dubbeldam and Visser, 1987; Witkovsky et al., 1973) whereas the rest of the body is mapped to the tectum. Following data from pigeons and mallards, it would be interesting to use physiological recordings to establish whether this holds true for the crocodilians, and such potential similarities could underscore the shared archosaurian phylogeny of the crocodilians and birds.

Fig. 1. Transverse section through the brainstem of a juvenile American alligator (*Alligator mississippiensis*) processed for the metabolic enzyme cytochrome oxidase. The principal sensory nucleus of the trigeminal nerve appears as a bi-lobed rounded structure at the lateral edge of the section. Cb, cerebellum; NMotV, trigeminal motor nucleus; PrV, principal trigeminal sensory nucleus; TrSpMes, spino-mesencephalic tract. Structures are identified based on the Cajal preparations of Huber and Crosby (1926).


Cb
NMotV
PrV
TrSpMes
1 mm

In a similar vein, efforts are underway to identify the visual Wulst, a distinctive structure found on the dorsal pallium of the telencephalon of many bird species (Karten et al., 1973) and examine its thalamic connections using a combined electrophysiological and tract-tracing technique (Fig. 2). Presumably a somatosensory area of the pallium might be found rostral to the visual areas (Pritz and Northcutt, 1980). Although the mammalian neocortex is has a columnar organization with primary sensory areas distributed across the laminar surface, the forebrains of reptiles and birds have the relatively thin pallium as the outermost neuronal layer. Areas homologous to the primary sensory areas of the mammalian neocortex are found with a large protrusion into the lateral ventricle known as the dorsal ventricular ridge (DVR) (Medina and Reiner, 2000; Northcutt and Kaas, 1995). Due to its relative location, it was long thought to be homologous to the basal ganglia (Herrick, 1910; Johnston, 1915); however, it contains neurons receiving projections from the thalamus, interneurons, and descending neurons that project to the brainstem and spinal cord – all qualities of the mammalian neocortex. These similarities have been examined in birds (Karten, 1969) and some reptiles (Bruce and Butler, 1984; Manger et al., 2002) but are not clearly understood within crocodilians, especially in respect to the physiological response properties of the telencephalic neurons. It is possible that through identification of sensory areas within the DVR of crocodilians a more complete understanding of the vertebrate brain could be ascertained. Only by understanding how the non-mammalian brain is organized can we gain insight into what features are novel and what are common between reptiles/birds and mammals and take the first steps towards understanding the evolutionary transformations that account for the neocortex.

Fig. 2. Photograph of the dorsal aspect of the brain of a juvenile American alligator. The green area on the right forebrain indicates a visually-responsive area of the pallium, the thin covering of the telencephalon (Tel). This is based on reconstructions from electrophysiological recordings of the right dorsal cortex of four alligators as part of a preliminary investigation of the visual Wulst of crocodilians. The blue area located laterally also has neurons that respond to visual stimulation; however these are located deeper within the telencephalon and may correspond to the pallial thickening. BS, brainstem; Cb, cerebellum; OB, olfactory bulb; OT, optic tectum; Sp, spinal cord; Tel, telencephalon; V, trigeminal ganglion.


Anterior
Posterior
OB
Tel
OT
V
Cb
BS
Sp
5 mm

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