Carotid Body: New Stimuli and New Preparations – *Invited Article*

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Abstract Beginning with the pioneering work of Heymans and collaborators in the 1930's, investigations into the role of the mammalian carotid body (CB) in the control of ventilation have attracted much attention. Progress for many years was restricted to the whole animal and organ level, resulting in characterization of the stimulus-response characteristics of the CB with its afferent nerve supply during exposure to chemostimuli such as low PO_2 (hypoxia), elevated PCO_2 (hypercapnia), and low pH (acidity). Major advances on the cellular and molecular mechanisms of chemotransduction occurred ~20 years ago with the use of freshly-dissociated CB preparations and single cell studies using patch clamp and spectrofluorimetric techniques. This review will focus on more recent advances based on novel preparations including co-cultures of isolated CB receptor clusters and dispersed sensory or autonomic neurons, thin CB tissue slice preparations, and transgenic models. These preparations have contributed significantly, not only to our understanding of the transduction and neurotransmitter mechanisms that operate in the CB during sensory processing, but also to the identification and characterization of novel CB stimuli such as hypoglycemia. Though the complexity of this remarkable organ still belies its tiny size, these recent advances are slowly unraveling the intricacies surrounding its ability to act as a polymodal detector of blood-borne chemicals and to alter its sensitivity to patterned stimuli.

Keywords Type I cells · Petrosal neurons · Carotid body co-cultures · Carotid body slice · Chemostimuli · Hypoglycemia

1 Introduction

The mammalian carotid body (CB) is a small chemoreceptor organ that senses chemicals in arterial blood and initiates compensatory reflex responses via increased afferent nerve activity, resulting in corrective changes in ventilation (Gonzalez

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et al. 1994). Aided by a rich vascularization, blood-borne chemical signals such as low PO_2 (hypoxia), elevated PCO_2 (hypercapnia), and low pH (acidosis) are transduced by endogenous chemoreceptor (type I) cells, which in turn release excitatory neurotransmitters onto terminals of the carotid sinus nerve (CSN). Our early understanding of the role of the CB in the control of ventilation was based on whole animal studies, aided subsequently by extracellular recordings of sensory discharge from the intact CSN. However, major breakthroughs on cellular and molecular mechanisms of chemotransduction occurred in the late 1980's and early 1990's with the application of patch clamp electrophysiological techniques and the monitoring of intracellular calcium signals in single isolated type I cells, following enzymatic dissociation of the CB. The reader is referred to other reviews for a comprehensive summary of these studies, as well as more recent advances using these 'reduced' single cell preparations (Gonzalez et al. 1994; Peers and Buckler 1995; Lopez-Barneo et al. 2001). A major focus of the present review is on more recent studies following the advent of other reduced preparations that came to fruition in the late 1990's and early 2000's. The following preparations have aided significantly in our understanding of sensory processing in the CB: (i) monolayer co-culture preparations of rat type I cell clusters and dissociated afferent (petrosal) and efferent (glossopharyngeal) neurons; (ii) thin living CB tissue slice preparations; and (iii) transgenic mice with knockout of specific protein targets. The main contributions from these preparations will be discussed in the following sections, and in some cases unifying results from the different preparations will be integrated for ease of presentation.

2 Co-Culture Preparations of Rat Carotid Body

In order to study synaptic transmission and afferent signaling in the CB, a co-culture model was developed and formally introduced in the late 1990's (Zhong et al. 1997). A central feature of this model is the development de novo of functional chemosensory units comprising type I receptor clusters and sensory (petrosal) neurons in monolayer cultures. A phase contrast photomicrograph from a typical co-culture of rat type I cells and petrosal neurons is shown in Fig. 1A. As is evident from this figure, in some cases both the neuronal soma and type I cell cluster may be fortuitously juxtaposed, thereby increasing the chances for successful contacts. In preparing these co-cultures, the usual first step is to plate dissociated CB cells such that islands or clusters of type I cells, arising from incomplete dissociation of the tissue, settle on the culture substrate. During the first few days in culture background cells including fibroblasts and other non-neuronal cells proliferate, while the type I cell islands (and contiguous glial-like type II cells) remain relatively stable even though type I cells do have the capacity for division (Nurse, 2005; Pardal et al. 2007).

The co-culture is then initiated a few days later by adding an overlay of enzymatically-dissociated neurons from the petrosal ganglia. Consequently, the co-culture contains a random distribution of type I clusters (2–20 cells) and individual

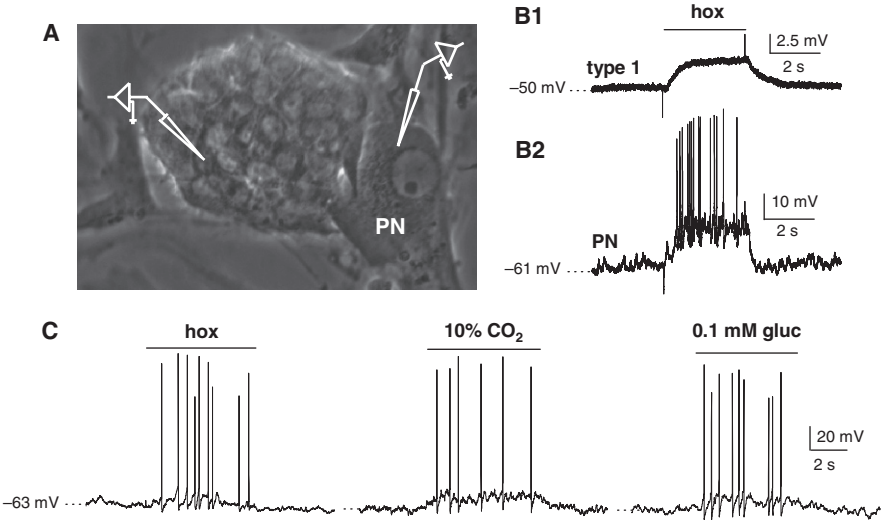

Fig. 1 Sensory transmission in co-cultures of rat type I cells and petrosal neurons. (**A**) Phase contrast photomicrograph of a typical co-culture containing a type I cluster and juxtaposed petrosal neuron (PN). Simultaneous dual perforated patch recordings can be obtained from both a presynaptic type I cell (within the cluster) and nearby postsynaptic PN as illustrated by the electrode placements in A. (**B**) Fast application of a hypoxic stimulus ($PO_2 \sim 5$ mmHg) caused a depolarizing receptor potential in the type I cell that was coincident with a suprathreshold chemoexcitatory response in the adjacent PN. (**C**) Recording from a functional PN in co-culture showed that the same chemosensory unit could be excited by three stimuli, i.e. hypoxia (*left*), isohydric hypercapnia (10% CO_2, pH=7.4; middle), and low glucose (0.1 mM; *right*)

neurons within a monolayer (Fig. 1A). Though not easily visible under phase contrast microscopy, neurofilament-positive processes extend from the petrosal cell bodies and ramify over the background cell monolayer, sometimes contacting type I clusters (Zhong et al. 1997). The key feature is that functional synaptic interactions often develop between outgrowing petrosal neurites and neighboring type I clusters, or between petrosal somas and contiguous type I cells. The main advantage of the preparation is that both presynaptic (i.e. type I cells) and postsynaptic (i.e. petrosal neuron) elements are clearly visible under phase or differential interference contrast optics and can therefore be conveniently studied by electrophysiological and/or imaging techniques (Fig. 1A). This contrasts with another commonly-used in vitro model consisting of the *intact* CB-attached sinus nerve preparation, where access and visibility at the synaptic sites are severely hampered, and recording of afferent activity is limited to propagating action potentials rather than synaptic potentials (Nurse 2005; Kumar and Bin-Jaliah 2007).

It should also be noted that as a result of incomplete dissociation, many type I cells in culture will have retained contacts with their normal type I cell neighbors as initially present in situ. The preservation of these contacts is advantageous because, unlike the situation for completely dissociated single type I cells, autocrine and paracrine interactions as well as electrical coupling can contribute

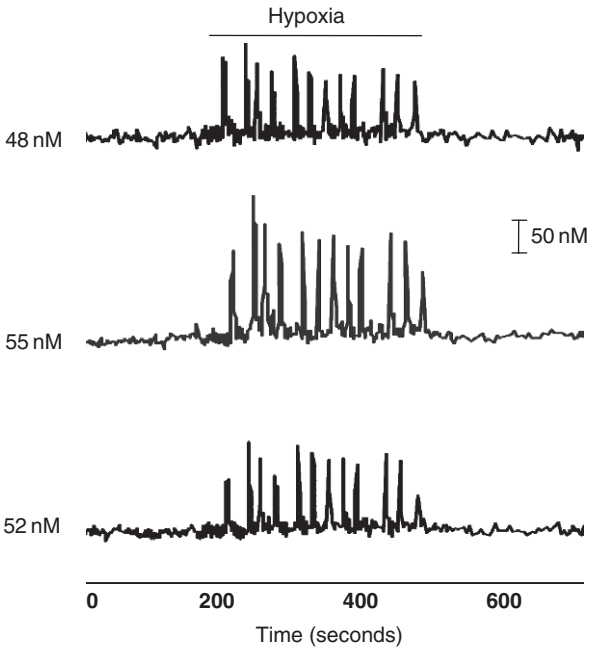

Fig. 2 Simultaneous ratiometric fura-2 intracellular Ca^{2+} measurements from type I cells within a cluster. Traces show responses from 3 non-contiguous cells within a cluster of ~12 cells before, during, and after exposure to hypoxia (PO_2 ~15 mmHg). Note intracellular calcium spikes or oscillations in each cell during hypoxia, and periods of apparent synchronous spike activity. Cell 1 (*upper trace*) was approximately 4 cell diameters away from cells 2 (*middle trace*) and 3 (*lower trace*); data from Dookhoo and Nurse, unpublished observations

normally to sensory processing under these monolayer conditions (Nurse 2005; Eyzaguirre 2007). Though not studied in detail, it is also conceivable that coordinated Ca^{2+}-signaling mechanisms that lead to synchronized activity may occur within type I cell clusters, as has been described in pancreatic ß-cells. For example, within larger clusters spontaneous Ca^{2+}-dependent action potentials have sometimes been recorded from a type I cell (Nurse 2005). More recently, our preliminary fura-2 imaging experiments have revealed intracellular Ca^{2+} spikes or oscillations in several cells within a cluster (Fig. 2), though it remains to be determined whether cell-cell interactions are required for this phenomenon.

A more recent and novel CB co-culture preparation was developed to permit studies of the role of autonomic efferent innervation on type I cell function (Campanucci et al. 2006). In this case, dissociated cholinergic and nitric oxide (NO)-positive autonomic neurons that are embedded in the glossopharyngeal and carotid sinus nerves were co-cultured with type I cell clusters. It is unclear whether *bona fide* synaptic contacts develop under these conditions, but these glossopharyngeal (GPN) neurons appear to have an 'attraction' for type I cells and form intimate, functional associations in culture (Campanucci et al. 2006).

In spite of the success of these models, the preparation of these co-cultures is both tedious and labor intensive, and therefore not amenable for routine use in most laboratories. Clearly, a readily available source of type I cells, for example an appropriate propagating cell line, would greatly facilitate studies on CB function. A particularly attractive recent development offers much promise in this area, with the discovery of glial-like stem cells in the adult rat CB (Pardal et al. 2007).

Under certain culture conditions, these stem cells form multipotent, self-renewing colonies and, dramatically, could differentiate into clones of type I cells with the same complex chemoreceptor properties of native, mature type I cells in situ.

3 Electrophysiological Experiments on Carotid Body Co-Cultures

Since its introduction the co-culture preparation of rat type I cells and petrosal neurons has been employed to uncover several neurotransmitter and neuromodulatory mechanisms that operate in the rat carotid body (Nurse 2005). In the majority of experiments reported to date, a depolarizing response obtained during perforated-patch recordings from petrosal neurons during application of the sensory stimulus has been used as a 'reporter' of functional connectivity with juxtaposed type I clusters (Fig. 1A,B). In some cases, simultaneous dual pre- and post-synaptic recordings have been obtained from a functional chemosensory unit (Zhang et al. 2007). An example is illustrated in Fig. 1B, where exposure to hypoxia caused type I cell depolarization associated with a depolarization and burst in spike activity in the adjacent neuron. Interestingly, as illustrated in Fig. 1C, the *same* chemosensory unit in co-culture could process several sensory modalities including hypoxia, hypercapnia, and the novel CB stimulus, low glucose (Pardal and López-Barneo 2002a). The fact that the neuronal afferent response to these chemostimuli could usually be blocked by a combination of purinergic (e.g. suramin) and nicotinic (e.g. mecamylamine) receptor blockers, and by low Ca^{2+}/high Mg^{2+}-containing extracellular solutions, provided compelling evidence for chemical transmission involving co-release of ATP and ACh from type I cells (Zhong et al. 1997; Zhang et al. 2000; Nurse 2005). Because in a few cases, blockade was not complete, other mechanisms including the additional co-release of 5-HT and/or electrical transmission, could not be ruled out (Zhang et al. 2000). Pharmacological characterization of the purinergic receptors expressed in identified chemosensory neurons in co-culture suggested contributions from both $P2X_2$ and $P2X_3$ subunits (Prasad et al. 2001). Importantly, $P2X_2$ and $P2X_3$ subunits were located at petrosal afferent terminals apposed to type I cells in the rat CB in situ using confocal immunofluorescence (Prasad et al. 2001). Confirmation of this prediction, together with complementary pharmacological studies in the isolated CB-sinus nerve preparation in vitro (Zhang et al. 2000), laid the cornerstone for the validity of the co-culture method as a reliable technique for studying CB function. Moreover, the subsequent demonstration that the hypoxic ventilatory response and hypoxia-induced sensory discharge in the sinus nerve were severely impaired in P2X2- (but not P2X3-) knockout mice provided compelling evidence for a central role of the purinergic $P2X_2$ subunit in sensory transmission (Rong et al. 2003). The co-culture model has also helped in uncovering autocrine/paracrine roles of CB neuromodulators. For example, endogenous release of 5-HT and GABA was found to enhance and inhibit CB chemosensory function respectively, via presynaptic G-protein coupled 5-HT_{2A} and $GABA_B$ receptors on type I cells (Nurse 2005).

In a more recent co-culture model of type I cells and efferent nNOS-positive glossopharyngeal (GPN) neurons, another potential role of ATP in mediating feedback inhibition was uncovered (Campanucci et al. 2006). In this study, application of ATP activated P2X receptors on juxtaposed GPN neurons, leading to type I cell hyperpolarization that was prevented by the NO scavenger, carboxy-PTIO. This raises the possibility of a dual excitatory/ inhibitory role of ATP during CB chemoexcitation. In the inhibitory pathway, released ATP may activate P2X receptors on nearby GPN terminals, causing an increase in intra-terminal Ca^{2+} that leads to activation of nNOS and NO synthesis/release.

4 Carotid Body Slice Preparation

Another key development in the late 1990's was the CB slice preparation that was elegantly exploited in several studies carried out in the laboratory of Professor José López-Barneo (Pardal et al. 2000; Pardal and López-Barneo 2002a,b). The earliest version of the CB slice preparation provided useful information on cholinergic receptive sites in the CB, but the small size of the type I cells combined with the use of intracellular electrodes resulted in low resting potentials and unstable recordings (Eyzaguirre 2007). In the more recent versions, the whole CBs are first collected in ice-cooled O_2-saturated Tyrode's solution and then embedded in a 3% (wt/vol) low-melting point agarose gel. After rapid cooling, the agarose block is glued to the stage of a vibratome or tissue vibroslicer, and 100–150 μm thick slices are cut with standard razor blades (Pardal et al. 2000; Pardal and López-Barneo 2002a,b). The slices may be used shortly after preparation or, more commonly, after culture for 24–48 h in DMEM medium supplemented with 10% fetal bovine serum (Pardal et al. 2000). The morphological appearance of a typical thin rat CB slice, viewed within a few hours after tissue section, is illustrated in Fig. 3A. After 24-48 h in culture, cell aggregates representing type I glomeruli become more easily discernible on the superficial aspects of the slice as discrete entities from the surrounding tissue, and are more accessible for cellular studies (Pardal et al. 2000; Fig. 3B). These glomeruli are immunopositive for tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine biosynthesis, confirming their identity as type I cells (Fig. 3C). The major advantages of the slice preparation are: (i) avoidance of enzymatic and mechanical dissociation of the tissue (as in dissociated cell culture preparations), resulting is better preservation of the cellular physiology and morphology; (ii) minimal disruption of cell-to-cell contacts among neighboring type I and/or type II cells, allowing intercellular interactions to be studied; (iii) the ability to perform high-resolution patch clamp techniques on single cells within the slice using infra-red DIC optics; and (iv) the ability to apply non-invasive techniques such as carbon fiber amperometry to monitor secretory activity from single, 'intact' type I cells. For example, such tissue slice preparations have been used to confirm the role of K^+ channel inhibition, membrane depolarization, and voltage-gated Ca^{2+} entry in hypoxia-induced catecholamine (CA) secretion from intact type I cells present

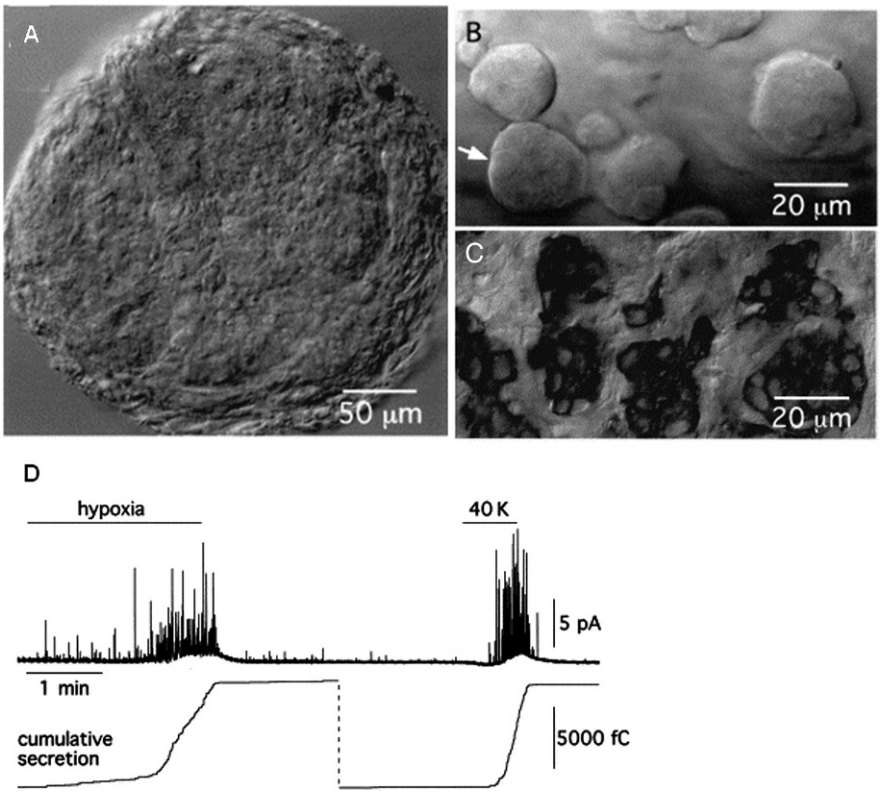

Fig. 3 Appearance and secretory function of thin slices of rat carotid body. Low power micrograph of a thin carotid body slice soon after sectioning (A). Note prominent clusters or glomeruli containing type I cells after culture of the slice for ~72 h (**B**). In fixed slices, these clusters are immunopositive for the catecholaminergic biosynthetic enzyme, tyrosine hydroxylase (**C**). In (**D**) carbon fiber amperometry was used to detect quantal catecholamine (CA) secretion from a type I cell in a slice preparation, following exposure to acute hypoxia (PO_2 ~ 20 mmHg; *upper trace*). Secretion was also evoked by the depolarizing stimulus high extracellular K^+. By integrating the area (i.e. charge) under the amperometric spikes, an estimate of total cumulative CA secretion during stimulus application was obtained (*lower trace*). This figure was modified from Pardal and López-Barneo (2002a; Figs. 1,5), and reproduced here with permission

within glomeruli (Pardal and López-Barneo 2002a; Fig. 3D). This preparation has also been used to shed light on a controversial issue arising from the use of dispersed cells as to whether hypoxia-induced closure of Ca^{2+}-dependent K^+ (BK) channels contribute to the PO_2 sensitivity of resting rat type I cells. While blockers of BK channel (e.g. TEA and iberiotoxin or IBTX) failed to affect membrane potential and intracellular Ca^{2+} levels in single isolated rat type I cells (Buckler 2007), these agents did indeed stimulate CA (Pardal et al. 2000) and ATP (Buttigieg and Nurse 2004) secretion from rat CB tissue slices. Because IBTX also stimulated CA secretion as determined by HPLC from clusters of cultured rat type I cells (see

Nurse 2005), it appears that at least some BK channels are open under resting (normoxic) conditions when rat type I cells are present in clusters or glomeruli, but may not be, when present as single dissociated cells. While the importance of isolated single-cell studies has been amply demonstrated, the above studies emphasize the additional need for studying type I cells within their native clustered arrangement for a complete understanding of CB function.

5 The Carotid Body as a Glucosensor: Contributions from Slice and Co-Culture Preparations

The slice preparation played a pioneering role in expanding our appreciation of the CB as a polymodal chemosensor and, particularly, in providing the initial evidence that type I cells may act as direct glucosensors (Pardal and López-Barneo 2002b). Though the CB has long been proposed to participate in glucose homeostasis, carbon fiber amperometric studies using the slice preparation provided the first compelling evidence that low glucose stimulated CA release from type I cells, an effect that was abolished by blockers of voltage-gated Ca^{2+} channels (Pardal and López-Barneo 2002b). Similarly, in fresh CB tissue slices low glucose was found to stimulate Ca^{2+}-dependent secretion of a key excitatory CB neurotransmitter, ATP (Zhang et al. 2007). In more recent electrophysiological studies on CB slices and cultured type I cells, hypoglycemia induced a depolarizing receptor potential due to activation of background cationic Na^{+}-permeable channels, possibly from the transient receptor potential C family (Garcia-Fernández et al. 2007; Zhang et al. 2007). These studies provided evidence that low glucose also caused inhibition of a voltage dependent K^{+} conductance and interacted additively with low PO_2, consistent with the involvement of separate transduction pathways.

The main conclusions derived from the CB slice model, concerning the mechanisms of glucosensing and the interaction between PO_2 and low glucose, have received strong support from the co-culture model. For example, in simultaneous recordings from a type I cell and juxtaposed petrosal neuron in co-culture, physiological hypoglycemia caused type I cell depolarization and an increase in petrosal sensory discharge (Zhang et al. 2007). An important common feature in physiological studies on the thin CB slice and co-culture preparations is that all chemostimuli, including low glucose, have *direct* access to the type I cells when applied to the bathing solution. As is evident from the above discussion, these two experimental approaches have led to the simplest conclusion that the action of low glucose on type I cells is direct. This contrasts with the conclusions from other studies based on the superfused, intact CB-sinus nerve preparation in vitro (Kumar and Bin-Jaliah 2007). In these studies, low glucose solutions had no effect on sensory discharge in the isolated whole CB-sinus nerve preparation in vitro, even though insulin-induced mild hypoglycemia caused hyperventilation in the whole animal that was prevented by CB denervation (Kumar and Bin-Jaliah 2007). Though these studies supported a role of the CB in the counter-regulatory response to hypoglycemia, they argue

for an *indirect*, rather than a direct, action of low glucose on CB receptors. The reasons for these discrepancies still await final resolution. However, in view of the negative findings, it is important that there be independent verification that glucose concentrations at the chemoreceptive sites are comparable to those in the bathing solution in these superfused (or perfused) in vitro whole CB preparations.

6 Transgenic Mouse Models

Another recent and important development in the carotid body field is the use of transgenic mouse models. One key contribution, i.e. the use of $P2X_2$ and $P2X_3$ single and double knockout mice to demonstrate the central role of ATP and the $P2X_2$ subunit in CB sensory transmission (Rong et al. 2003), has already been mentioned above. The reader is referred to a recent comprehensive review on the contributions of transgenic models to our understanding of CB function (Ortega-Sáenz et al. 2007). These models have included transgenic mice deficient in putative O_2-sensor proteins, e.g. NADPH oxidase, hemeoxygenase-2, succinate dehydrogenase (SDHD), and HIF-1α. While these models will become increasingly important in our quest to understand CB function, interpretation of the results will need caution because of the possibility of compensatory developmental changes, and the fact that the transduction pathways (e.g. O_2-sensitive K^+ channel subtypes) may vary among different species.

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