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The Characteristics of the Electrovomeronasogram: Its Loss following Vomeronasal Axotomy in the Garter Snake

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Abstract

Electrovomeronasogram (EVG) recordings were made from adult garter snakes, *Thamnophis sirtalis*. Stimulation of vomeronasal epithelium with a stimulus prepared from prey, earthworm electric shock secretion (ESS), evoked EVG response in a dose-dependent manner. The magnitude of the EVG response to ESS was remarkably larger than *n*-amyl acetate and glutamate, which elicited insignificant responses, supporting the idea that the vomeronasal system is differentially sensitive to liquid delivery of biologically significant chemical stimuli. Fourteen days following vomeronasal axotomy, the magnitudes of the EVG responses of animals which received bilateral axotomy without cauterization or with cauterization was –0.19 ± 0.07 mV or –0.05 ± 0.02 mV respectively, compared with the normal EVG response of –0.41 ± 0.10 mV. The epithelia of animals which received bilateral axotomy without cauterization exhibited remarkable degeneration of the bipolar neurons. Maximal depletion of bipolar neurons occurred in the epithelia denervated with cauterization, though the difference between cell densities in vomeronasal neuron layers in these epithelia was not statistically significant. The present results clearly indicate that the fewer neurons the epithelium contains, the smaller EVG response it generates, suggesting that the receptor neurons are the primary origin of EVG responses.

Introduction

Nasal chemosensory neurons detect and transmit information about the chemical environment to the central nervous system. The initial process of chemoreception involves interaction of stimuli with the bipolar neurons in the sensory epithelium. The electro-olfactogram (EOG) and electrovomeronasogram (EVG) responses involve slow transepithelial potential changes evoked by stimuli and measured extracellularly at the olfactory and vomeronasal epithelial surface respectively.

EOG measurements are used as tools in evaluating the excitatory or inhibitory actions of chemical stimuli (Menevse *et al.*, 1977; Goldberg *et al.*, 1979; Senf *et al.*, 1980; Simmons and Getchell, 1981; Mackay-Sim *et al.*, 1982; Schafer *et al.*, 1984; Mackay-Sim and Kesteven, 1994; Li *et al.*, 1995). It is widely accepted that the EOG represents chiefly a summation of generator potentials in the olfactory neurons (Takagi and Yajima, 1964, 1965; Booth *et al.*, 1981; Simmons and Getchell, 1981; Evans and Hara, 1985; for a review see Getchell, 1986). However, a contribution by cells other than olfactory neurons to generating EOG responses has also been reported (Shibuya, 1964; Evans and Hara, 1985). For example, Evans and Hara (1985) reported that the EOG responses evoked by HCl in sensory and non-sensory epithelia were indistinguishable from each other, indicating that receptor neurons are not likely to be their primary origin.

EVG responses have also been recorded from the epithelium of some terrestrial vertebrates such as amphibians (*Rana*) (Müller, 1971), tortoises (Tucker, 1963; Graziadei and Tucker, 1970; Kruzhalov and Boîko, 1987), the box turtle (Graziadei and Tucker, 1970), lizards (Müller, 1971), snakes (Inouchi *et al.*, 1993), mice (Müller, 1971) and humans (Monti-Bloch *et al.*, 1994), enhancing our understanding of stimulus specificity and functional properties of the vomeronasal organ in terrestrial vertebrates. In spite of the usefulness of EVG measurements for peripheral activation of the vomeronasal epithelium, the source of the observed change in electrical activity that occurs during stimulation of the vomeronasal epithelium with appropriate odorants has not yet been investigated. Denervation of vomeronasal neurons results in their degeneration (Barber and Raisman, 1978; Wang and Halpern, 1982a,b). To investigate whether the EVG reflects summed neural activity of vomeronasal bipolar neurons, the effect of vomeronasal axotomy was studied by comparing evoked responses in the vomeronasal epithelium in intact versus deafferented vomeronasal organs. In the same

animals, histological observations of the vomeronasal epithelia were correlated with electrophysiological results.

To investigate the characteristics of the EVG further, we measured the EVG response of garter snakes to several stimuli, including biologically relevant chemoattractants delivered as liquid. The present results provide evidence that the EVG response results from summed vomeronasal neural activity and have further characterized the EVG response to liquid delivery of stimuli with respect to stimulus specificity.

Materials and methods

EVG recordings

Adult garter snakes, *Thamnophis sirtalis*, weighing 34–131 g, were obtained from commercial suppliers and maintained at 22°C. Twenty-three animals were used in the present study. However, data taken from one animal were excluded because the magnitude of the EVG response to electric-shock-induced earthworm secretion (ESS), which is the most reliable stimulus for the snake vomeronasal organ, was widely dispersed even under the same experimental conditions. The methods were essentially similar to those described previously (Inouchi *et al.*, 1993). In brief, snakes were anesthetized with urethane (0.1 g/100 g body wt) and immobilized with *d*-tubocurarine chloride (2.5 mg/100 g body wt). Prior to an experiment, each snake was tested to ascertain whether it would accept earthworm prey. Only snakes that ate the prey were used in the present study.

The vomeronasal epithelium was exposed by removing the medial palate and mushroom body. EVG recordings were made from the surface of the vomeronasal epithelium, with an Ag–AgCl electrode bridged via a glass capillary (tip diameter 50–100 μm) filled with Ringer's solution in 1% agar, amplified with a DC preamplifier and displayed on a pen recorder. The EVG induced by a stimulus was recorded from the center portion of the dorsal neuroepithelium. A reference electrode (chloride-coated silver wire) was placed in the mouth. The magnitude of the EVG response was measured as the height of the phasic displacement from the baseline.

Vomeronasal nerve section

For surgery, animals were cooled prior to anesthetization with an i.m. injection of Brevital Sodium (Eli Lilly & Co., Indianapolis, IN; 1.5 mg/100 g body wt). The snakes were attached to a glass plate. Overlying scale, skin, connective tissue and cartilage were removed from the cranium, exposing the vomeronasal nerves, which were cut using forceps. Following nerve section with or without cauterization, the cavity was filled with gelfoam (Upjohn, Kalamazoo, MI). Subsequently, this area was covered with a piece of adhesive tape. The animals recovered from surgical anesthesia within 1–3 h. In the experiments shown in Figures 1B–E and 3, EVG recordings were made in snakes subjected to unilateral or bilateral vomeronasal nerve lesions 2 weeks prior to the recording session. Two weeks is the optimal interval for maximal degeneration of receptor neurons in the vomeronasal epithelium (Wang and Halpern, 1982a).

Histological observations

For histological observations of epithelial degeneration, the animals under anesthetic were perfused i.c. with saline, followed by Bodian's fixative (Bodian, 1936). The heads were stored in fixative for several days, decalcified for 2–3 days in DECAL containing 9.5% HCl and catalytic calcium ion chelating agent (Omega Chemical Corp., Cold Spring, NY), followed by washing under running tap water for 3 h. Tissues were dehydrated and embedded in Paraplast (Oxford Labware, St Louis, MO). Twelve micrometer paraffin sections were cut in the horizontal plane. The mounted sections were impregnated with 1% Protargol S (without copper) for 18–24 h at room temperature. Tissues on the slides were reduced and fixed according to the method reported by Bodian (1936, 1937).

The morphological observations were supplemented by a quantitative estimation of changes in cell population within cell columns. Because the receptor neurons and undifferentiated cells were morphologically indistinguishable in this study, an undifferentiated cell layer was defined as being the 10 cells at the base of each column (Wang and Halpern, 1982a). After the vomeronasal nerve is cut, however, the basal cells proliferate (Wang and Halpern, 1982a). Therefore, there is a possibility that the counts of the receptor cells in the cell columns in the lesioned animals are overestimates.

Chemical stimulation

Li and Halpern (1994) reported that liquid delivery of earthworm and goldfish wash to the vomeronasal epithelium increased the spontaneous firing of mitral cells in the accessory olfactory bulb, but vapor delivery to the vomeronasal epithelium had no effect. In the present study, therefore, the epithelia were stimulated by aqueous solutions. The irrigating and stimulating solutions were delivered to the vomeronasal epithelium through a micropipette attached to capillary tubing at a rate of 2 ml/min. Gravity was used to deliver a constant stream of these solutions. To eliminate any nonspecific effects of the mechanical stimulation, the epithelium was irrigated with Ringer's solution for at least 2 min just prior to the 2 s application of the stimulating solution. At least 2 min rinsing using Ringer's solution was interposed between successive stimulations to wash out the stimulating solution. All experiments were carried out at room temperature.

Preparation of figures

Stained sections of the vomeronasal organ were captured with a Nikon Microphot-SA (Nikon, Tokyo, Japan) with a Digital photographic camera (model DKC-5000, SONY,

Ichinomiya, Japan) into a Power Macintosh computer and stored using Adobe Photoshop software (Adobe Systems, Inc., San Jose, CA).The final figure was assembled with Canvas software (Deneba System, Inc., Miami, FL).

Solutions

Snake Ringer's solution consisted of (in mM): 156 NaCl, 5 KCl, 3 $CaCl_2$, 1.8 $MgCl_2$, 10 HEPES–NaOH, pH 7.4. *n*-Amyl acetate and L-glutamic acid were dissolved in Ringer's solution. ESS was prepared as described elsewhere (Jiang *et al.*, 1990). In brief, 300–400 ml of earthworms rinsed with Ringer's solution were placed into the cone of an electric stimulator. An electric current from a 9 V battery was applied as 10 s bursts with 60 s intershock intervals. During this time, the earthworms secreted viscous, cloudy, mucus-like fluid containing chemoattractants for snakes. Because of its high viscosity, the fluid was collected in a beaker by rinsing the earthworms with Ringer's solution. Stock solutions of ESS containing proteins at average concentrations of 8.5 mg/ml were stored in a refrigerator until use.

Chemicals

n-Amyl acetate, L-glutamic acid and Protargol-S were purchased from Fisher Scientific (Fair Lawn, NJ), Sigma Chemical Company and Johnson Matthey S.A. (Paris, France) respectively. All other reagents were of the highest grade commercially available.

Results

Responsiveness of the EVG to stimuli

Figure 1A shows typical records of the EVG response to chemoattractants of varying concentrations. The response was a negative slow voltage transient, reaching a peak at onset of stimulation and declining to the baseline on cessation of the stimulus. In this study the peak height of the response was taken as the magnitude of the response. As seen in the figure, the magnitude of the EVG response to ESS increased with increasing concentration, indicating that these responses are generated in a dose-dependent manner.

Figure 2A shows the relative magnitudes of EVG responses to stimuli where the magnitude of the response to ESS was taken as unity. The EVG response to ESS was greater than those to the other stimuli used. As shown in the figure, the relative magnitudes of the EVG responses to *n*-amyl acetate and L-glutamate were <0.05. The present results suggest that ESS produces an EVG response which is more distinct and reliable than those to the other stimuli examined.

Effects of vomeronasal axotomy on the epithelium and EVG response

ESS was selected as a stimulus in this experiment because it was the most effective stimulus of the vomeronasal

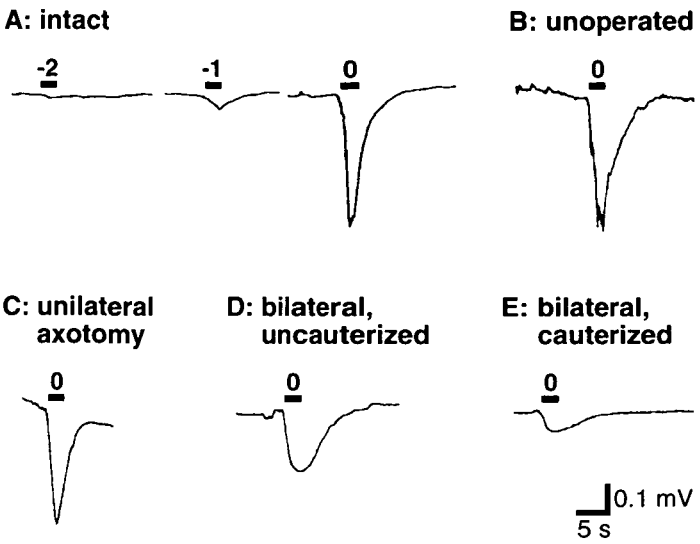

Figure 1 **(A)** EVG to ESS recorded from snakes which received no surgery. **(B–E)** EVG to ESS recorded from the epithelia on unoperated side (B) and operated side (C) of the animals which received unilateral axotomy, or from those of animals which received bilateral axotomy without (D) or with (E) cauterization. Dilutions (10^{-2}–10^{-0}) are as indicated. The stock solution of ESS (0) contains 8.5 mg/ml protein. Solid bars above traces indicate duration of exposure to the stimuli.

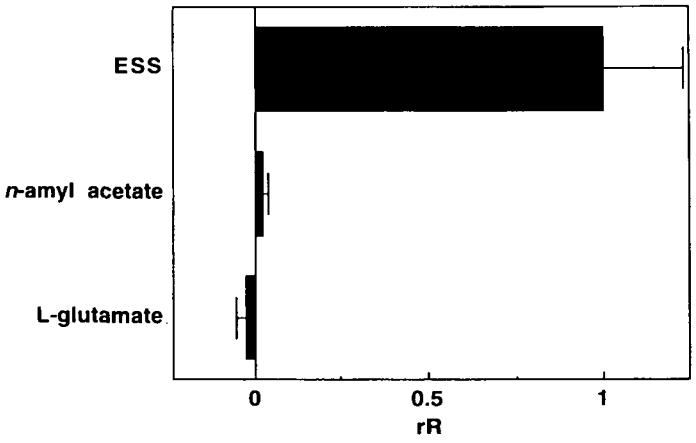

Figure 2 Relative magnitudes of the electrovomeronasogram to various stimuli. The magnitude of the EVG response was calculated relative to those to ESS. Points represent the means ± SEM of data obtained from at least five preparations. The mean magnitude of the EVG response to ESS was −0.41 ± 0.10 mV. The ESS solution contained 8.5 mg/ml protein. Concentrations of both *n*-amyl acetate and glutamate were 10^{-2} M.

epithelium (Figure 2). Using behavioral methods, Schulman *et al.* (1987) reported that snakes with olfactory or sham bilateral nerve lesions discriminated ESS from distilled water following surgery whereas snakes with vomeronasal nerve lesions did not, suggesting that ESS is detected by the vomeronasal organ.

The EVG responses to ESS recorded from epithelia of garter snakes which had undergone unilateral or bilateral

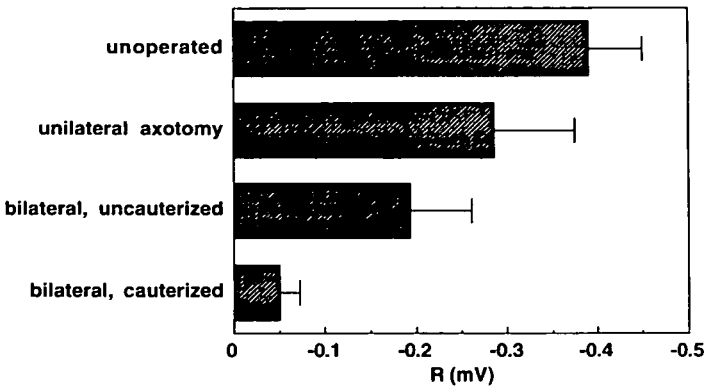

Figure 3 Effects of vomeronasal axotomy on EVG response to chemoattractants. Fourteen days following axotomy, EVG responses to ESS were recorded from the epithelia on the unoperated (unoperated) and operated sides of the animals which received unilateral axotomy (unilateral axotomy), or from those of animals which received bilateral axotomy without (bilateral, uncauterized) or with (bilateral, cauterized) cauterization. Points represent the means ± SEM of data obtained from at least six preparations.

axotomy are illustrated in Figure 1B–E. The EVG response recorded from epithelium on the unoperated side of the snake which received unilateral axotomy was practically the same as that recorded from epithelium of the animal receiving no surgery (Figure 1B,C), indicating that unilateral axotomy does not affect the EVG response recorded from epithelium on the unoperated side. As shown in Figure 1D,E, the EVG responses recorded from epithelia after bilateral axotomy reduced in amplitude and showed relatively moderate peaks.

Figure 3 shows the magnitude of the EVG response to ESS after axotomy. The magnitudes of the EVG responses recorded from animals which underwent bilateral axotomy without cauterization or with cauterization was –0.19 ± 0.07 (n = 12) or –0.05 ± 0.02 mV (n = 12), respectively, compared with a normal EVG response of –0.41 ± 0.10 mV (n = 7). The magnitude of the EVG responses was significantly reduced by bilateral axotomy especially with cauterization.

Figure 4 shows the histological changes of the sensory epithelium 14 days following axotomy. The vomeronasal epithelium of garter snakes contains three types of cells (basal undifferentiated, bipolar and supporting cells) that are segregated into distinct layers (Wang and Halpern, 1980). The undifferentiated and supporting cells are housed in a vertically oriented columnar matrix (Figure 4A). In the epithelia on the operated side of snakes which received attempted unilateral axotomy, slight degeneration of the receptor neurons was seen (Figure 4B), suggesting that either the lesions were not extensive or that regeneration had occurred. Similar results were observed in three other preparations. This slight degeneration of the receptor neurons, however, is consistent with the insignificant reductions of the magnitude of the EVG response (Figure 3). In the epithelia of animals which received bilateral axotomy without cauterization, remarkable degeneration of the neurons was generally observed (Figure 4C), suggesting that the bilateral lesion approach was more successful in sectioning the vomeronasal nerves. Similar results were observed with another 11 preparations. Maximal depletion of the neurons occurred in the epithelia denervated with cauterization (Figure 4D), thus preventing regeneration (n = 12).

Figure 5 presents the cell density in control and denervated vomeronasal epithelia. Though the receptor neurons and undifferentiated cells were morphologically indistinguishable from each other in 12-μm-thick sections using Bodian stain for light microscopy, the results shown in Figure 5 reflect two of the events occurring in the denervated vomeronasal epithelium: (i) the population of supporting cells is practically unchanged or slightly increased following axotomy; and (ii) the density of the cells in the estimated receptor neuron layer decreased following axotomy. Bilateral axotomy caused a significant decrease in the cell density of estimated receptor neurons, whereas unilateral axotomy produced a minimal decrease in the population of vomeronasal neurons,

Figure 6 plots the magnitude of the EVG response to ESS against the cell density calculated from control and denervated vomeronasal epithelia. There is a good correlation between both values (r = 0.90).This result, together with the results shown in Figures 3–5, clearly indicates that the fewer neurons the epithelium contains, the smaller the EVG response generated by ESS, suggesting that the EVG response is dependent on the functional receptor neuron population.

Discussion

The vomeronasal epithelium of animals with attempted unilateral nerve section did not demonstrate an altered response to stimulation with ESS on the lesioned side. This result was, in fact, quite surprising. However, histological examination of the vomeronasal epithelium indicates that complete degeneration of bipolar neurons was not present in the epithelia on the operated side of snakes that underwent attempted unilateral axotomy. Wang and Halpern (1980) reported that incomplete vomeronasal axotomy, which did occur in some of their cases, resulted in degeneration of bipolar neurons in restricted cell columns only. Thus, the failure to observe complete degeneration of the bipolar neuron layer may be due to partial nerve lesion. However, the present results cannot rule out the possibility that regeneration contributed to the presence of a large number of bipolar neurons on the side operated on.

In contrast, bilateral vomeronasal nerve lesions led to both a striking reduction in the EVG to ESS stimulation and a marked depletion of the neuronal population in the vomeronasal epithelium. The greater reduction in both EVG

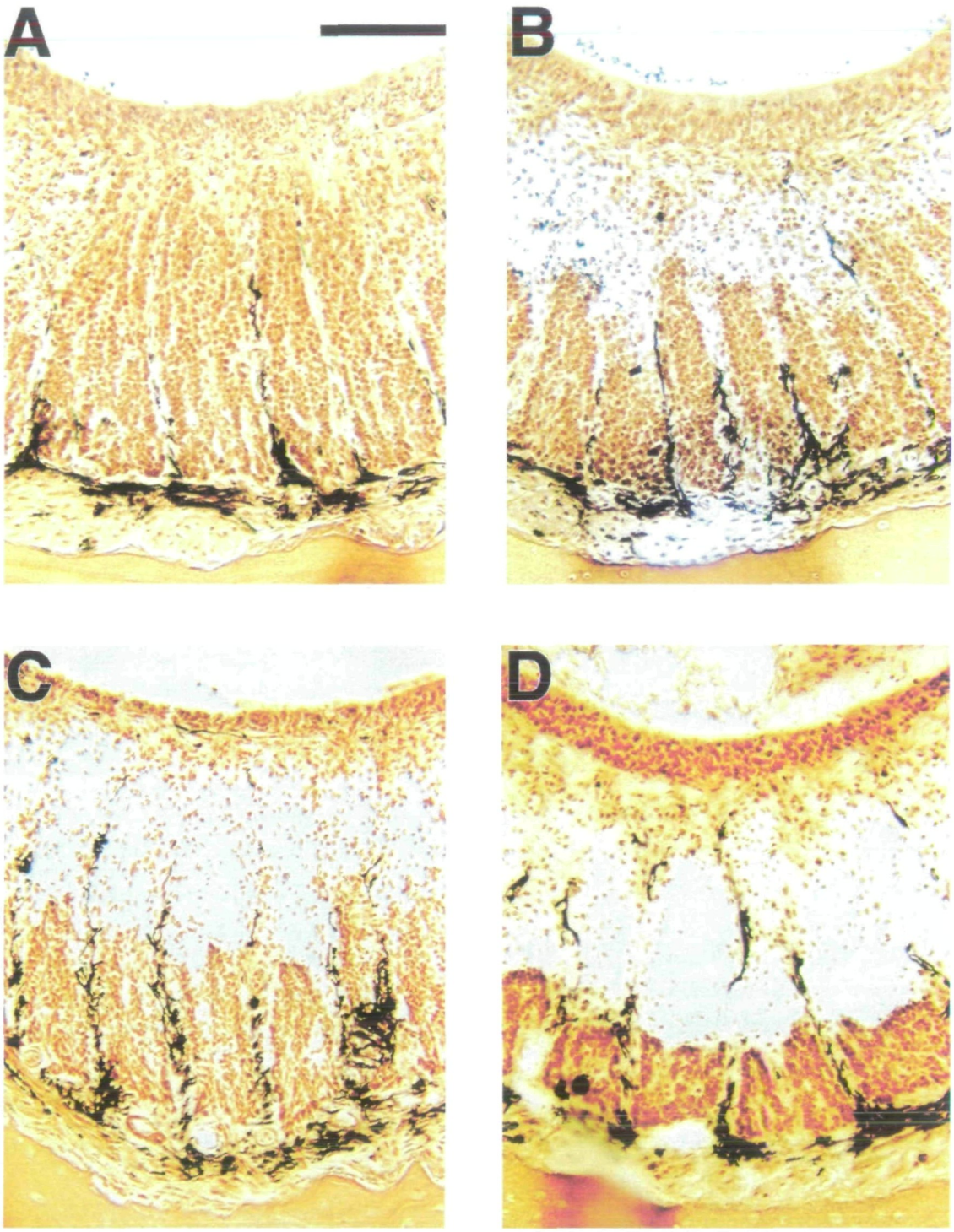

Figure 4 Photomicrograph of Bodian-stained horizontal sections (12 μm) of snake vomeronasal sensory epithelia 14 days following axotomy. **(A)** Intact vomeronasal epithelium. Note that there are no empty spaces in the epithelium. **(B)** Epithelium unilateral to attempted vomeronasal axotomy. Slight degeneration of bipolar neurons was observed. **(C, D)** vomeronasal epithelium of snakes receiving bilateral axotomy without (C) and with cauterization (D). Remarkable degeneration of the neurons was generally observed. Note the large gaps in the epithelium of the snake with cauterized nerves (D). Scale bar = 100 μm.

and bipolar neurons in animals with their vomeronasal nerves cauterized as compared with animals with bilateral lesions but not cauterized suggests that when the cut ends are not cauterized, regeneration may occur. Wang and Halpern (1982b) demonstrated the regeneration of the vomeronasal epithelium in animals with unilateral lesions

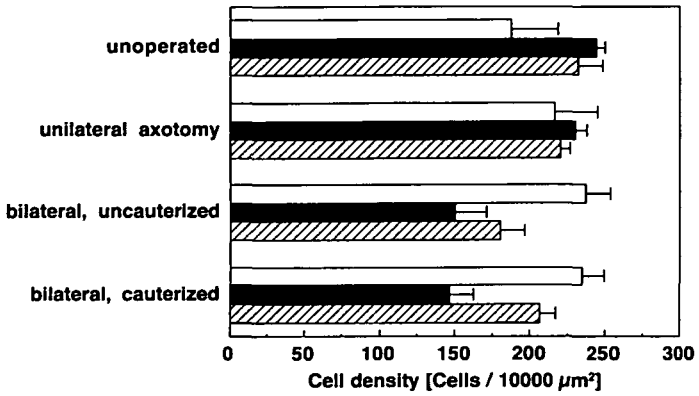

Figure 5 Density of vomeronasal cells following denervation as observed in histological sections of a cell column. Unoperated and unilateral axotomy: cell columns in the epithelia unoperated side and operated side of the animals which received unilateral axotomy respectively. Bilateral, uncauterized and bilateral, cauterized: cell columns in the epithelia of animals which received bilateral axotomy without and with cauterization respectively. Points represent the means ± SEM. At least five cell columns in a section of at least four animals in a category were calculated and averaged. Open, dark shadowed and cross-hatched bars represent cell densities of supporting cell layer, estimated receptor neuron layer and undifferentiated cell layer respectively. The counts of the receptor cells in the cell colums in the lesioned animals may be overestimated (see Materials and methods for details). The mean area of supporting cell, neuron and undifferentiated cell layers in a column of unoperated animals were (in 10000 μm^2) 0.170 ± 0.022, 1.214 ± 0.065 and 0.470 ± 0.043 (mean ± SEM) respectively.

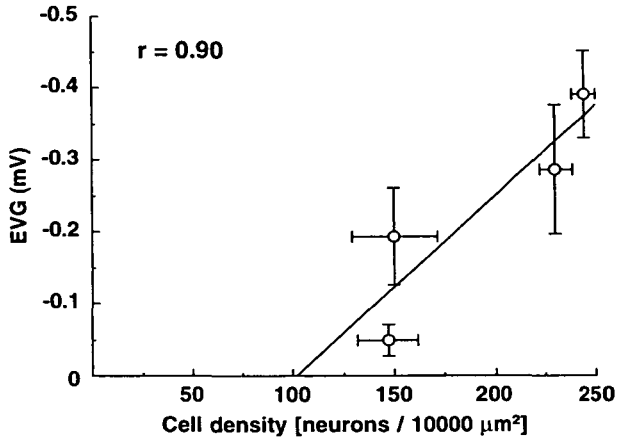

Figure 6 Correlation between the magnitude of the EVG response to ESS and the cell density of estimated receptor neuron layer. Values were taken from Figures 3 and 5. Points represent the means ± SEM.

and no cautery of the cut ends. The greater numbers of bipolar neurons in the vomeronasal epithelium of snakes with bilateral lesions but no cautery could be the result of regeneration, as suggested above for the unilateral lesions, although 2 weeks is a relatively short time to observe significant regeneration (Wang and Halpern, 1982b). Conversely, the process of cauterization itself might have increased the number of nerve fibers severed after some of the fibers were severed by the forceps.

We have demonstrated that the fewer neurons the vomeronasal epithelium contains, the smaller the EVG response it generates (Figure 6). This finding suggests that the receptor neurons are the primary origin of EVG responses. As far as we know, this is the first study that investigates the dependence of the EVG on neural activation.

Acknowledgements

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