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Miniaturization in plethodontid salamanders (Caudata: Plethodontidae) and its consequences for the brain and visual system

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In seven species of plethodontid salamanders (*Desmognathus ochrophaeus, Eurycea bislineata, Plethodon cinereus, Batrachoseps attenuatus, Hydromantes italicus, Thorius narisovalis* and *Bolitoglossa subpalmata*), absolute and relative volumes of the eye, the brain, major regions of the brain, and regions containing the major visual and visuomotor centres (i.e. thalamus, praetectum, tectum and tegmentum mesencephali), and the density and number of neurons in these regions were determined. The seven species range from moderately large to extremely small in body size and from the smallest to the largest genome sizes found in terrestrial salamanders.

The following processes were observed in miniaturized salamanders with intermediate to large genome and cell sizes (*Batrachoseps, Thorius*) as compared to small and medium-sized salamanders with small genome and cell sizes: (1) increase in the relative size of the brain, from 3.9 to 12.4% of head volume; (2) reduction in relative size of the ventricles from 10.9 to 5.8% of brain volume; (3) increase in relative volume of those brain regions containing the major visual and visuomotor centres from 29.2 to 37% of brain volume; (4) increase in volume of grey matter relative to white matter, from 33.2 to 44.4% of midbrain volume; (5) increase in volume of tectal relative to tegmental grey matter, from 54.8 to 76.8% of total midbrain volume; (6) increase in neuron packing density in the regions containing the visual centres, from 16 to 31.5%. Because of these compensatory processes, *Thorius*, the smallest species with a head 1/27 and a brain 1/9 the size of that of the largest one, *Hydromantes*, has 1/3 as many central visual neurons (58 000 *vs.* 187 000).

Some of these processes found in miniaturized salamanders, such as increase in tectal cell density, also occur in large salamanders with very large genome and cell sizes, viz. in *Bolitoglossa* (25%) and *Hydromantes* (29%). Thus, increase in genome size and cell size seem to pose functional problems similar to miniaturization; both cases involve an increase in cell size relative to overall organismal structure.

KEY WORDS:—Brain – visual system – salamanders – miniaturization – genome size – cell size – cell number – scaling – evolution – constraints.

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INTRODUCTION

Evolutionary morphologists lately have increased their efforts to delineate the role played by intrinsic, or structural, factors in the evolution of morphological diversity. One especially important outcome of these studies has been the proposal that such factors play a prominent role in defining the range and nature of morphologies attained, independent of extrinsic, environmental factors (Gould, 1980; Lauder, 1982; Roth, 1982; Wake, 1982; Wake, Roth & Wake, 1983; Roth & Wake, 1985).

Perhaps the most conspicuous morphological characteristic of an organism that may constrain diversity is size. For virtually any structure or functional unit there is a range of absolute size over which it can function properly; outside this range, function will be impaired in the absence of an appropriate design change. Thus, by looking at size extremes of living organisms, one may identify those specific features that are most limiting for a particular body plan.

The relation between body size and structure is well demonstrated for numerous anatomical systems in vertebrates (Calder, 1984; Went, 1968). Indeed, many phylogenetically significant changes in morphology can be considered scaling effects, or size-dependent structural changes that maintain functional efficiency (Schmidt-Nielsen, 1975, 1984). For the most part, however, previous studies have emphasized the limits imposed by size *increase* while ignoring the limits imposed by, and compensatory adjustments evolved in response to, size *decrease*. This is especially surprising in view of the predominant importance of extreme size decrease, or miniaturization, to macroevolutionary phenomena such as the origin of morphological novelty (Hanken, 1983, 1984, 1985).

In this paper we address the consequences of miniaturization in vertebrates for the brain and sense organs, especially the visual system. We focus on several members of one of the most successful and diverse vertebrate lineages, the lungless salamanders of the family Plethodontidae which comprises 27 genera and at least 220 extant species, or about 60% of all living salamanders (Duellman & Trueb, 1986; Lombard & Wake, 1986). Miniaturization has evolved several times independently within this group which includes some of the smallest living vertebrates (Wake & Lynch, 1976; Wake & Elias, 1983).

Initial earlier studies, which focused on the Mexican genus *Thorius*, revealed

pervasive consequences of miniaturization for gross cranial morphology, especially with regard to the packaging of nervous and sensory components in a tiny head (Hanken, 1983, 1984; Roth *et al.*, 1983). Negative allometry of both eye and brain size relative to head size, which characterizes both intraspecific and interspecific comparisons among plethodontid salamanders, results in these organs predominating in miniaturized forms, seemingly at the expense of the adjacent skull which is noticeably reduced. Additional changes involve brain and eye shape, as well as their spatial position relative to each other and to adjacent sense organs—the olfactory organ and inner ear.

Subsequent studies, which considered a wide size-range of species in several genera, identified additional scaling effects correlated with miniaturization that affect several aspects of eye size and internal structure (Roth *et al.*, 1983; Linke *et al.*, 1986). These include increased relative eye volume; thicker retinal layers, especially the layer of retinal ganglion cells; a larger proportion of cones (*vs.* rods); and denser packing of the receptors and other cells.

These studies further revealed that a more comprehensive understanding of the intrinsic constraints on miniaturization would require detailed knowledge about cell size and number. For example, the size of cells, including neurons, varies greatly among vertebrates (Olmo, 1983). Thus, vertebrates with the same brain volume may have very different numbers of neurons owing to differing cell size. If minimum cell number is a limiting factor in neural design and function, then comparisons based on gross brain size alone are at best incomplete and at worst erroneous. Compared to most vertebrates, salamanders are small and on average possess small brains relative to body size. On the other hand, they have the largest genomes and cells of all terrestrial vertebrates; those of plethodontid salamanders are especially large (Hally *et al.*, 1986; Sessions, 1984). Accordingly, in salamanders, cell size may be a critical parameter that limits miniaturization, especially with regard to the proper function of sense organs and sensorimotor parts of the brain. That is, the number of neurons in miniaturized species with large or medium-sized cells may lie at, or close to, the minimum level for neural or sensory function. This line of reasoning leads directly to two questions. First, how small can a vertebrate become without the loss of sensory and motor functions essential for survival, e.g. sufficient visual acuity and coordination of feeding behaviour? Second, how large can genomes and cells become without adverse effects on function?

In this study, we present comparative data on gross size and proportions, cell density and cell number of the eye and especially the brain with which we address the phenomenon of miniaturization of the visual system in plethodontid salamanders. We emphasize the fate of the visual system, because all salamander species studied here depend heavily on vision (e.g. for feeding). For this reason, detailed morphometric studies were performed on those brain regions that contain the principal primary and secondary visual and visuomotor centres, viz. the thalamus of the diencephalon, the praetectum, and the midbrain with the tectum opticum and the tegmental regions.

MATERIAL AND METHODS

One species each from seven plethodontid genera were included in the analysis (Table 1; Fig. 1). Together they represent a wide range of body and head sizes,

TABLE 1. Species studied. Snout-vent length (SVL, in mm) was measured to the posterior end of the vent. Genome-size measurement (GS, in pg DNA per nucleus per haploid genome) for *Eurycea* is from Moreschalchi (1975); all others are from Sessions (1986)

Species	SVL	GS
Subfamily Desmognathinae		
Desmognathus ochrophaeus	32.5[a]	14.6 ± 0.3
Subfamily Plethodontinae		
Tribe Hemidactyliini		
Eurycea bislineata	38.0[b]	20.8
Tribe Plethodontini		
Plethodon cinereus	41.6[c]	22.3 ± 0.6
Tribe Bolitoglossini		
Supergenus *Bolitoglossa*		
Bolitoglossa subpalmata	44.6[d]	68.9 ± 2.0
Thorius narisovalis	26.6[e]	25.2 ± 0.5
Supergenus *Hydromantes*		
Hydromantes italicus	52.2[f]	76.2 ± 0.6
Supergenus *Batrachoseps*		
Batrachoseps attenuatus	44.6[a]	37.0 ± 2.3

[a]Roth, unpublished data. [b]Duellman & Wood, 1954. [c]Hanken, unpublished data. [d]Vial, 1968. [e]Hanken, 1985. [f]Lanza, 1952.

from relatively large plethodontid salamanders (*Hydromantes, Bolitoglossa*) to true miniatures (*Batrachoseps* and especially *Thorius*) which may reach sexual maturity at body sizes less than 15 mm snout-vent length (SVL). In addition, they represent all of the major lineages—subfamilies, tribes and supergenera—within the Plethodontidae, thus providing the opportunity for phylogenetic comparisons within this monophyletic group (Wake, 1966; Fig. 1).

Desmognathus ochrophaeus and *Eurycea bislineata,* both from eastern North America, are aquatic as larvae but semiaquatic as adults. *Plethodon cinereus,* also from eastern North America, lacks an aquatic larval stage and, instead, has direct development. All four remaining species—*Batrachoseps attenuatus* from

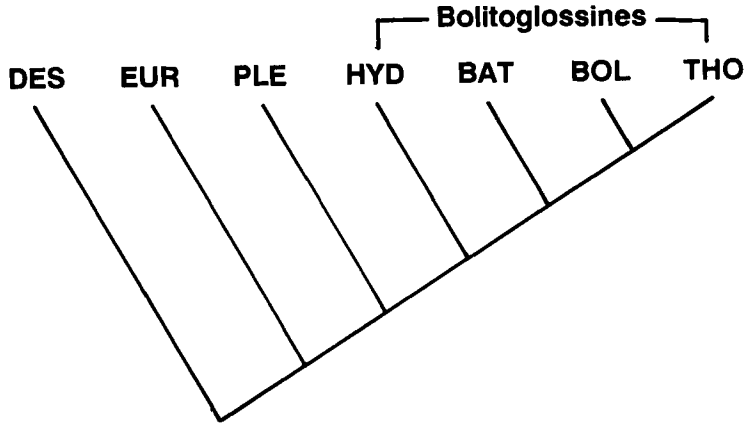


Figure 1. Hypothesis of phylogenetic relationship among the seven plethodontid species included in the analysis (adapted from Lombard & Wake, 1986).

California, *Hydromantes italicus* from Italy, *Thorius narisovalis* from Mexico and *Bolitoglossa subpalmata* from Costa Rica—are direct developers with a highly derived ontogeny ("ontogenetic repatterning", Roth & Wake, 1985). *Batrachoseps attenuatus* became secondarily elongated after miniaturization (Wake, personal communication). Thus, for this species, head size is a much more accurate indicator of miniaturization than is body size (Tables 1, 2).

Genome sizes of these species range from among the smallest (*Desmognathus*, 14.6 pg DNA per nucleus) to the largest (*Hydromantes*, 76.2 pg) found in plethodontid salamanders (Moreschalchi, 1975; Sessions, 1984).

The three non-bolitoglossine genera represent primitive outgroups with respect to the four bolitoglossines (Fig. 1). Based on such outgroup comparisons, both tiny head and body sizes (i.e. miniaturization) and large genome size are derived features within the tribe Bolitoglossini.

Determination of head, eye, brain and ventricle volumes

Salamander heads (five specimens per species) were fixed for 1–2 weeks in Bouin's solution modified according to Brasil-Dubosq (Romeis, 1968). They were decalcified in a solution of 3% hydrochloric acid/70% ethanol for 1–3 weeks, depending on head size. After being rinsed in tap water overnight, the heads were embedded in Histosec, cut into 20-μm serial sections, and silver-impregnated according to Palmgren (Romeis, 1968).

In order to determine volumes of the head, eyes, brain, ventricles and major brain regions, we drew equidistant cross sections of the eye (10–12 sections) and brain (30–50 sections) with a Zeiss camera lucida. Owing to differences in head, eye and brain sizes, the distance between sections varied between 20 μm (*Thorius*) and 300 μm (*Hydromantes*). Respective areas were measured using a graphics tablet (Summagraphics) coupled with a computer program.

In order to determine volumes of the major parts of the brain (i.e. telencephalon (forebrain), diencephalon, mesencephalon (midbrain), and rhombencephalon (cerebellum + medulla oblongata)), their boundaries were established, mainly according to Herrick (1948) (Fig. 2A). Although modern neuroanatomists (e.g. Naujoks-Manteuffel & Manteuffel, 1986) consider the praetectum to represent a distinct brain region located parallel to the fibres of the commissura posterior (Fig. 2B), the pretectal nuclei (nucleus praetectalis, nucleus Darkschewitsch) are difficult to distinguish without experimental manipulation, e.g. retrograde labelling of nerve cells with horseradish peroxidase. We, therefore, did not treat the praetectum as a separate region; the pretectal nuclei are incorporated into measurements of tectal parts of the mesencephalon. This procedure is justified because both pretectal nuclei are closely associated anatomically and functionally to the tectum (e.g. with regard to object identification (cf. Manteuffel, 1984, 1986; Roth, 1987)).

Boundaries of the brain areas measured are as follows.

Telencephalon-diencephalon. Ventrally, the boundary lies immediately caudal to the commissura anterior and the commissura hippocampi and rostral to the nucleus praeopticus; medially, the boundary runs rostral to the eminentia thalami and along the tractus amygdalo-thalamicus; dorsally, it lies rostral of the commissura habenulae.

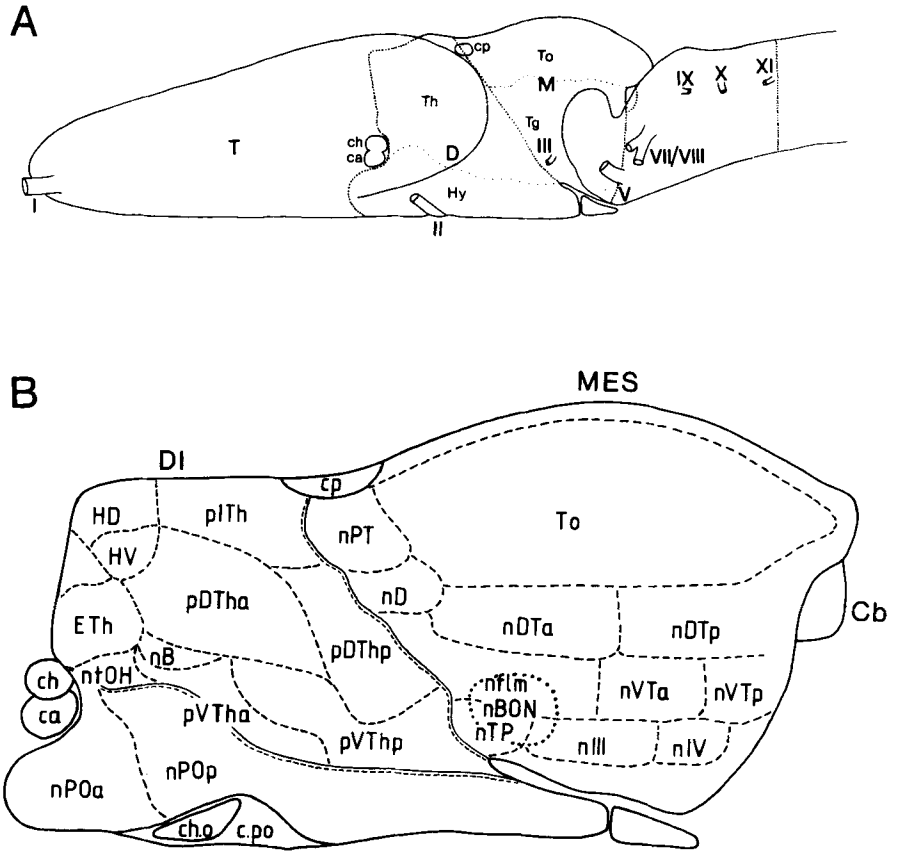


Figure 2.A. Salamander brain (lateral view). Boundaries between major parts of the brain are indicated by densely stippled lines. Borders between the thalamus and hypothalamus within the diencephalon, and between the tectum opticum and tegmentum inside the mesencephalon, are indicated by loosely stippled lines. B, Lateral view of the diencephalon, praetectum, and mesencephalon showing the distribution of centres, nuclei and commissures as revealed by normal and HRP-material.

Diencephalon-mesencephalon/praetectum. Diencephalic regions (e.g. pars intercalaris thalami, pars dorsalis thalami, pars dorsalis hypothalami) often are difficult to distinguish from adjacent mesencephalic regions (e.g. eminentia commissurae posterioris, n. tuberculi posterioris). To obtain reliable data, the boundary between diencephalon and mesencephalon was represented by a straight line drawn between the dorsally situated commissura posterior and the ventrally situated fovea isthmi. Inside the diencephalon, the border between thalamus and hypothalamus was drawn along the sulcus ventralis thalami (cf. Fig. 3A).

Mesencephalon-rhombencephalon. This boundary is constituted dorsally by the caudal end of the tectum, and medially by the commissura cerebelli, the rostral end of the n. cerebelli, and the caudal end of the tegmentum trigemini and tegmentum abducentis. Ventrolaterally, the beginning of the rhombencephalon is easily identified by the root of the trigeminal nerve. Ventromedially, however, the rhombencephalon is more difficult to identify. Here, a line along the dorsal

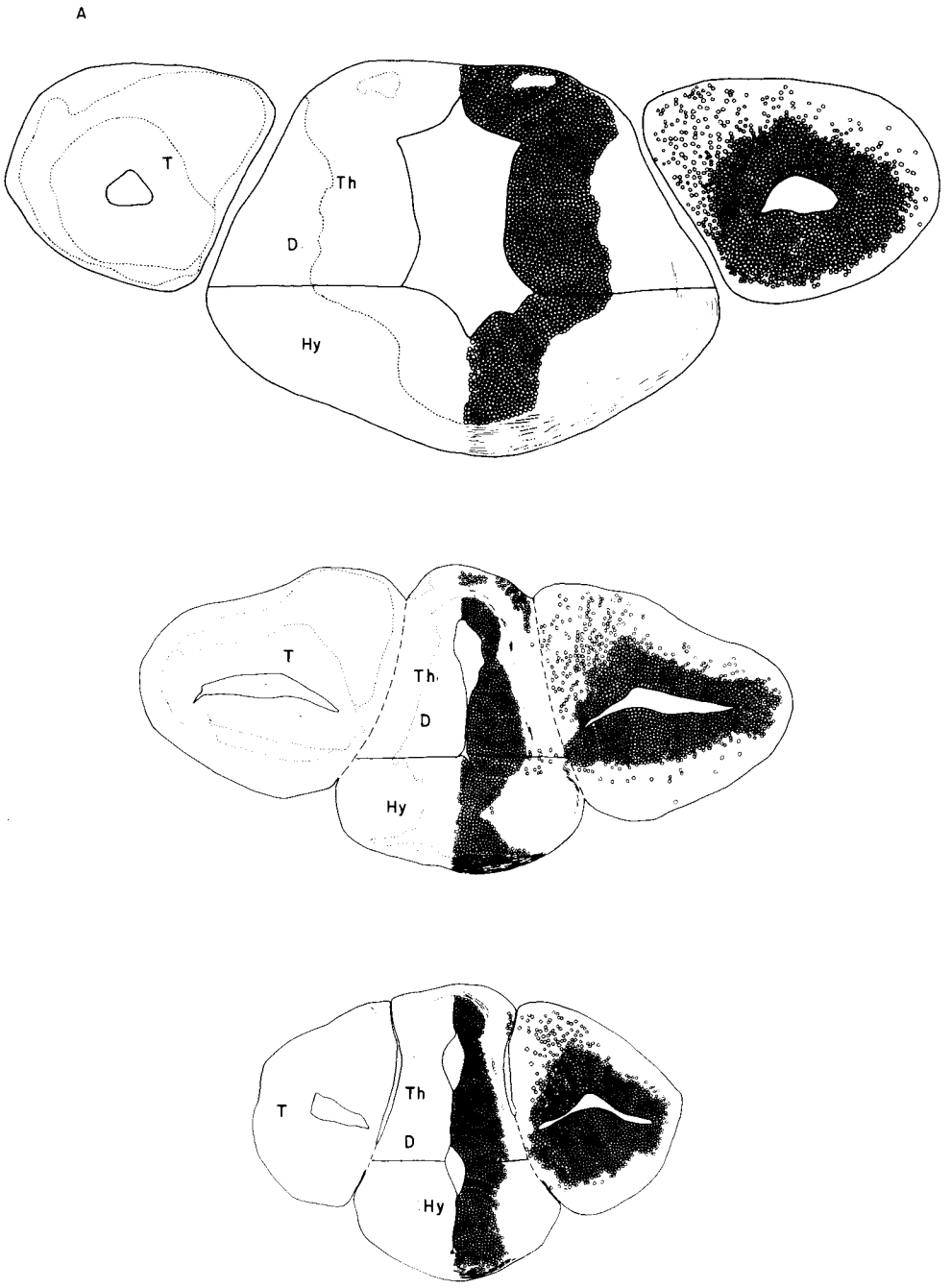
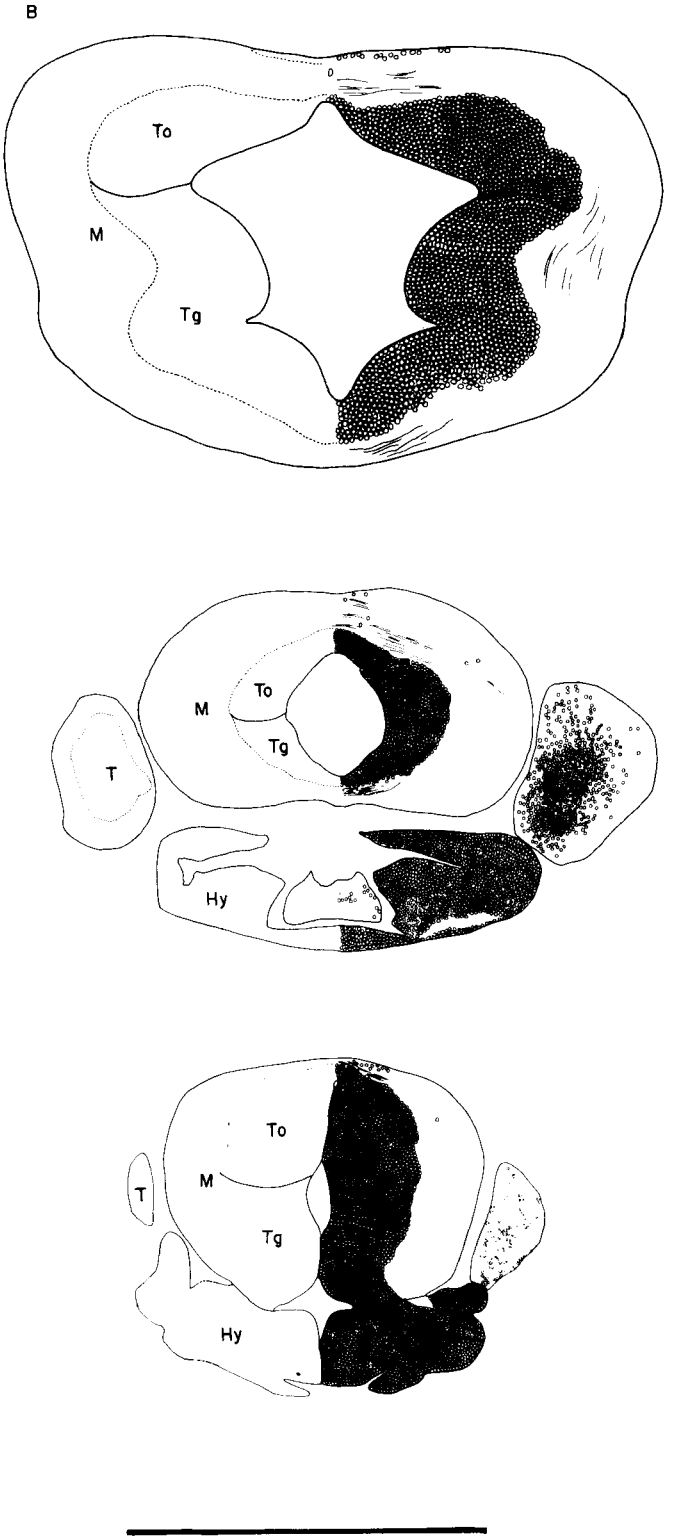


Figure 3.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edge of the massive longitudinal fascicles, which are easily identified in Palmgren-stained transverse sections, was used as the border between mesencephalon and rhombencephalon.

Determination of cell density and cell number in brain regions containing visual and visuomotor centres

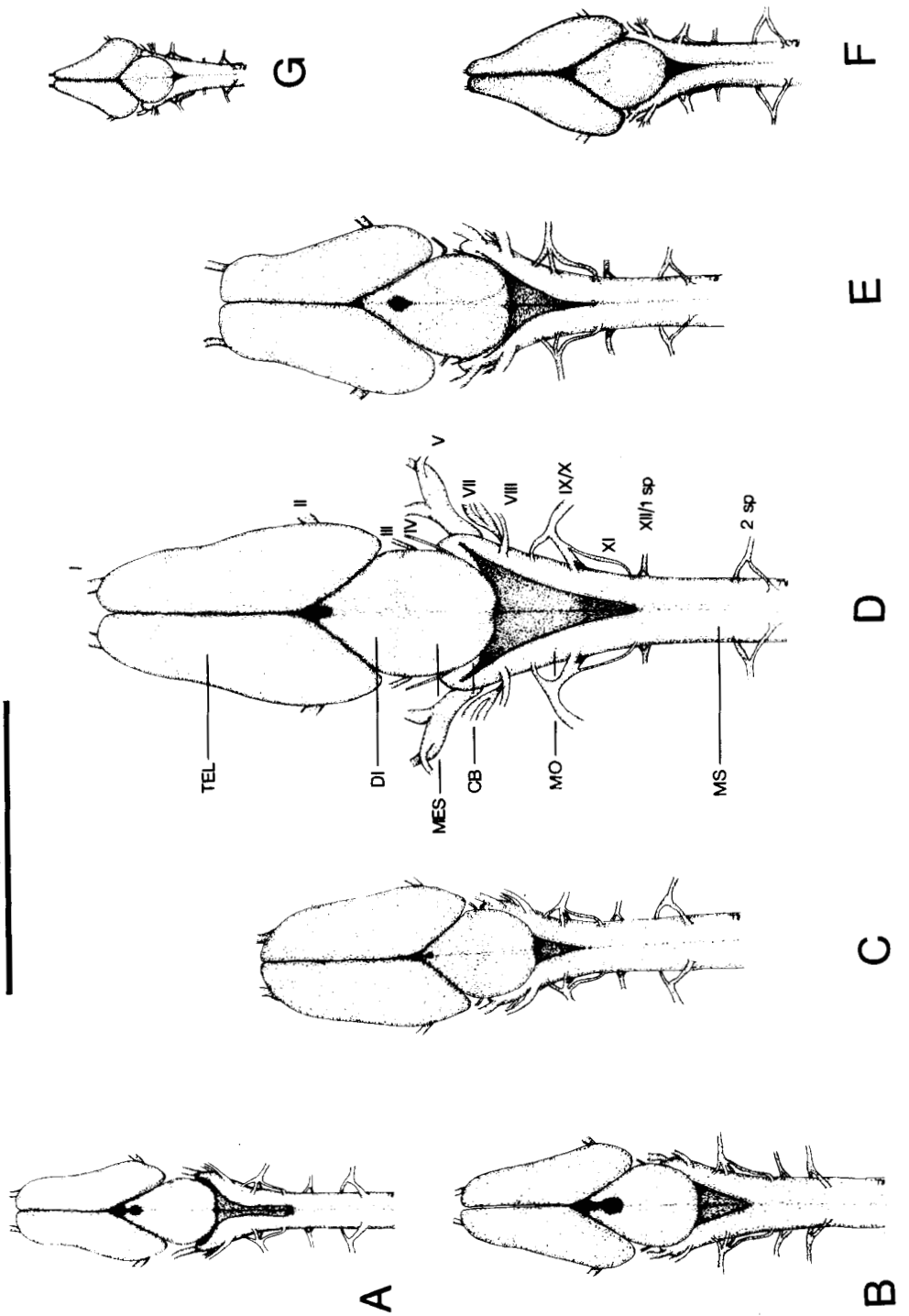
To control for shrinkage of neural tissue during fixation and staining and its effect on estimates of cell density and cell size, measurements were made on two types of brain-section material—one treated according to the Rapid-Golgi method, the other fixed with Bouin's solution (modified after Brasil-Dubosq) and stained after Palmgren as mentioned above. Golgi material apparently undergoes little shrinkage but is poorly suited for detailed morphometric studies; Palmgren material shrinks more but is excellent for morphometric studies. All procedures mentioned below were done in parallel with both types of histological preparations. Golgi material was embedded in Epon and cut either in 30-, 60- or 80-μm sections, according to brain size. Cell size was determined using Nomarski optics that clearly reveal the outlines of stained as well as unstained cells. Bouin-Palmgren material was cut in 20-μm sections.

Parts and nuclei of the diencephalon, praetectum and mesencephalon identified on the basis of normal and experimental material are illustrated in Fig. 2B. The centres and nuclei involved in processing of visual information, as revealed by neuroanatomical (HRP) preparations and electrophysiological recordings, are as follows (for further details see Roth, 1987). (1) Diencephalon: anterior and posterior pars dorsalis and pars ventralis thalami, nucleus Bellonci, possibly pars intercalaris thalami and nucleus praeopticus. (2) Praetectum: nucleus praetectalis profundus and superficialis, nucleus Darkschewitsch. (3) Tectum mesencephali. (4) Mesencephalic tegmentum: anterior and posterior nucleus ventralis thalami, nucleus fasciculi longitudinalis medialis, nucleus neuropili optici basalis, nucleus of the oculomotor nerve, nucleus of the trochlear nerve, nucleus isthmi.

To determine the volumes of the diencephalic and mesencephalic/pretectal regions mentioned above, the same morphometric method was used as for the whole brain. The ratio of white to grey matter within these regions was determined in the same way.

Measurements of cell density, diameter and number in the thalamus, tectum/praetectum and tegmentum were made for representative areas of dense (tectum) and loose (tegmentum) cell packing, with edge length between 65 and 110 μm depending on brain size. The number of cells within this standard area was counted at a magnification of × 460. The diameters of 25 cells per unit area were measured in both histological preparations. Three specimens were used for

Figure 3. Tracings from transverse sections through (A) the diencephalon, including caudal poles of the telencephalon, and (B) the mid-mesencephalon, of *Hydromantes italicus* (above), *Batrachoseps attenuatus* (middle) and *Thorius narisovalis* (below). Boundaries between the thalamus and hypothalamus within the diencephalon (at the level of the sulcus ventralis thalami), or between the tectum opticum and tegmentum within the mesencephalon, are indicated by horizontal lines; cellular layers are indicated on the right side. Scale bar = 1 mm.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each preparation type and for each species. Thus, a total of 42 brains was studied.

The correction formula, $N_V = N_A/D'' + T$, was used to calculate cell density. N_V is cell density (number of cells per μm^3), N_A is the number of cells counted in the standard area, D'' is the mean cell diameter within the standard area, and T is the section thickness (Abercrombie, 1946; Ball & Dickson, 1983). Mean cell numbers for the thalamus, tectum/praetectum, and tegmentum were obtained by multiplying the volume of each of these brain regions by its respective N_V-value. Regional cell density of each region was determined by dividing the volume of perikarya in that region (calculated by multiplying cell density, N_V, by mean cell volume) by the total volume of the region, expressed as a percentage.

In order to calculate the total cell density and cell number of the brain regions containing the visual centres, density values for the thalamus, tectum/praetectum and tegmentum were summed after each value was weighted by a factor proportional to the relative volume of its respective region.

Statistical comparisons of cell diameter and density used the two-tailed *t*-test.

RESULTS

Gross brain morphology

Gross brain morphology in miniaturized species (*Batrachoseps, Thorius*) is very different from that in larger taxa (Figs 3, 4). The forebrain is located more posteriorly and is triangular-shaped (*vs.* rectangular) in dorsal view. This is correlated with a substantial increase in the relative size of the eyes which, together with the nasal capsules, occupy most of the space in the rostral part of the head in these species. The telencephalon and diencephalon are more compressed. The rhombencephalon extends forward under the mesencephalon, such that the caudal poles of the forebrain and the auricles of the rhombencephalon almost contact each other. Consequently, the midbrain covers more of the rhombencephalic groove.

Head, eye and brain size

As one would expect according to Haller's rule of brain-body allometry (Rensch, 1959), among the seven species studied relative brain size generally increases as head (and body) size decreases (Table 2; Fig. 5). *Hydromantes*, the largest salamander of the series, has the relatively smallest brain, whereas *Thorius*, the smallest salamander within the series (its head volume is about one-thirtieth that of *Hydromantes*), has the relative largest brain. There are, however, seeming exceptions from Haller's rule. *Bolitoglossa*, for example, has a much

Figure 4. Brains of the seven plethodontid species studied; all are drawn to the same scale in dorsal view. A, *Desmognathus ochrophaeus*. B, *Eurycea bislineata*. C, *Plethodon cinereus*. D, *Hydromantes italicus*. E, *Bolitoglossa subpalmata*. F, *Batrachoseps attenuatus*. G, *Thorius narisovalis*. Major parts of the brain and roots of the cranial and first two spinal nerves are indicated for *Hydromantes*. The abducens nerve (VI), which exits the medulla oblongata ventromedially between nerves VII and IX, is not drawn. Scale bar = 5 mm.

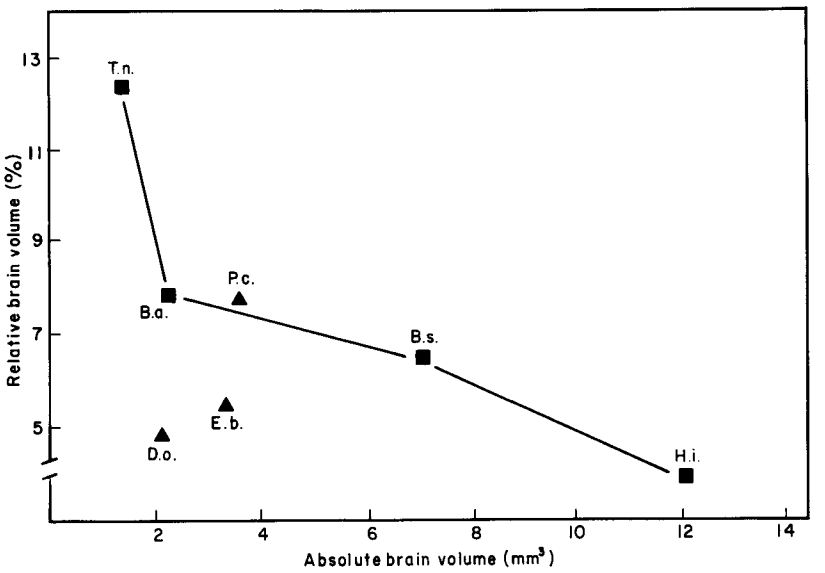

Figure 5. Relative brain volume (expressed as a percent of total head volume) as a function of absolute brain volume. ■, Bolitoglossine species (connected by a line); ▲, non-bolitoglossine species. Species are listed in the legend to Fig. 4.

larger brain than would be expected based simply on body and head size. And *Desmognathus,* which would be expected to have an intermediate relative brain size similar to that of *Eurycea,* has the second smallest brain in relative terms.

The pattern in relative eye size is still more complicated (Table 2; Fig. 6). *Thorius,* the smallest species, has by far the largest relative eye size, which one would expect from Haller's rule. But *Hydromantes* and *Bolitoglossa,* which might be expected to have the smallest eyes relative to head size, have the second- and fourth-largest eyes. In fact, all four bolitoglossine species have larger relative eye sizes than the three non-bolitoglossines. The relatively smallest eyes are found in *Desmognathus,* the third-largest salamander.

Size of the ventricles

Relative ventricle size (total ventricle volume divided by total brain volume) is generally proportional to body size (Table 3). *Hydromantes,* the largest species,

TABLE 2. Absolute volume (mm^3) of the head, eyes and brain (including ventricles), and relative volume of the eyes and brain expressed as percentage of head volume. Each value denotes the mean ± SE; $N = 5$. Species are arranged in order of increasing head size

Genus	Head	Eyes	Brain	Eye (%)	Brain (%)
Thorius	11.70±1.2	1.20±0.20	1.41±0.36	10.07	12.28
Batrachoseps	28.93±1.6	1.93±0.09	2.26±0.38	6.70	7.76
Plethodon	48.42±8.7	2.36±0.25	3.55±0.64	5.05	7.69
Eurycea	57.45±13.5	2.86±0.60	3.32±0.63	5.22	5.46
Desmognathus	59.97±20.0	2.09±0.51	2.12±0.71	4.24	4.76
Bolitoglossa	119.33±25.0	7.32±1.45	7.09±1.80	5.60	6.42
Hydromantes	313.00±7.8	21.80±1.32	12.14±0.61	7.00	3.88

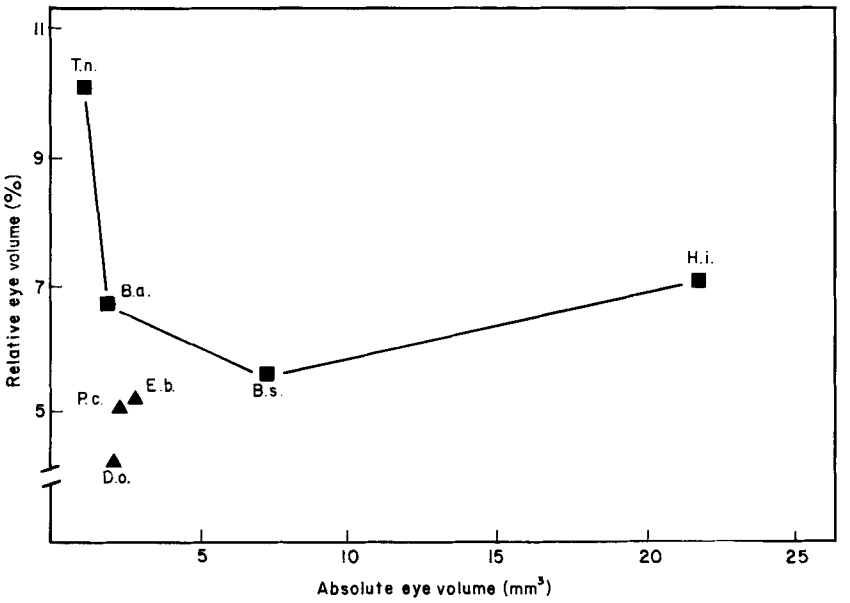

Figure 6. Relative volume of both eyes (expressed as a percentage of head volume) as a function of absolute eye volume. ■, Bolitoglossine species (connected by a line); ▲, non-bolitoglossine species.

has the largest relative ventricle size. It is followed by *Plethodon*, *Eurycea* and *Desmognathus*—the three non-bolitoglossine species which are of intermediate body size. *Thorius* and *Batrachoseps*, the two smallest species, have the smallest relative ventricle sizes (cf. Fig. 3). Surprisingly, *Bolitoglossa*, the second largest species, has the third-smallest relative ventricle size.

Sizes of major brain parts

There are large differences in the relative proportions of the major brain parts among the species studied (Tables 4, 5). *Hydromantes* and *Bolitoglossa* each have a relatively large forebrain and a relatively small diencephalon and midbrain.

TABLE 3. Absolute volume ($mm^3 \times 10^{-3}$) of the first and second (V1+2), third (V3), and fourth (V4) ventricles, total ventricular volume (VT), and total relative volume of the ventricles (V%) expressed as percent of total brain volume. Each value denotes the mean ± SE; $N = 4$ for *Batrachoseps* and *Eurycea*; $N = 3$ for the remaining species

Genus	V1+2	V3	V4	VT	V%
Thorius	44±8	47±5	12±1	103	7.6
Batrachoseps	60±24	49±18	22±9	131	5.8
Plethodon	155±59	171±81	71±3	397	10.9
Eurycea	104±35	182±57	82±2	368	10.6
Desmognathus	86±60	101±70	54±34	241	9.3
Bolitoglossa	134±98	208±103	115±34	457	8.3
Hydromantes	533±40	757±117	119±33	1,409	11.6

TABLE 4. Absolute volume (mm^3) of the telencephalon (TC), diencephalon (DC), mesencephalon (MC), diencephalon plus mesencephalon (DC+MC), and rhombencephalon (cerebellum plus medulla oblongata; RC) (without ventricles). Each value denotes the mean ± SE; $N = 5$

Genus	TC	DC	MC	DC+MC	RC
Thorius	0.44±0.12	0.20±0.03	0.16±0.04	0.36	0.18±0
Batrachoseps	1.02±0.09	0.36±0.02	0.32±0.03	0.68	0.33±0
Plethodon	1.61±0.19	0.49±0.03	0.49±0.03	0.98	0.50±0
Eurycea	1.02±0.20	0.50±0.06	0.58±0.05	1.08	0.42±0
Desmognathus	0.80±0.11	0.31±0.02	0.33±0.02	0.65	0.42±0
Bolitoglossa	3.63±0.50	1.06±0.09	0.83±0.10	1.89	1.06±0
Hydromantes	7.06±0.94	2.01±0.37	1.66±0.25	3.67	1.75±0

TABLE 5. Relative volume of the telencephalon (TC), diencephalon (DC), mesencephalon (MC), diencephalon plus mesencephalon (DC+MC), and rhombencephalon (RC), expressed as percent of total brain volume (without ventricles). Each value denotes the mean of five specimens per species

Genus	TC	DC	MC	DC+MC	RC
Thorius	45.1	21.5	15.5	37.0	14.1
Batrachoseps	50.1	17.9	15.9	33.8	16.0
Plethodon	51.9	15.9	16.1	32.0	16.2
Eurycea	42.7	20.9	18.0	38.9	17.7
Desmognathus	42.6	16.1	13.4	29.5	24.1
Bolitoglossa	54.6	16.5	12.7	29.2	16.2
Hydromantes	57.0	15.9	13.4	29.3	13.7

TABLE 6. Diameter (μm) of nerve cell perikarya within the tectum opticum (TO) and the tegmentum (TG) in Palmgren and Golgi preparations. Each value denotes the mean ± SE; $N = 3$. The overall mean of all four values for each species is on the right

Genus	Palmgren		Golgi		
	TO	TG	TO	TG	Mean
Thorius	11.0±0.2	10.5±0.4	12.7±0.9	12.9±0.5	11.8
Batrachoseps	10.6±0.4	11.7±0.4	13.4±0.2	14.1±0.7	12.5
Plethodon	8.9±0.3	9.3±0.2	10.1±0.7	9.8±0.6	9.5
Eurycea	8.4±0.4	9.5±0.2	9.0±0.3	9.3±0.3	9.1
Desmognathus	7.5±0.2	8.0±0.3	9.9±0.4	10.1±0.7	8.9
Bolitoglossa	12.9±0.8	12.4±1.3	15.1±0.2	15.8±0.2	14.1
Hydromantes	14.9±0.9	15.5±1.2	18.0±0.7	17.2±0.1	16.5

Thorius has a relatively small forebrain and a large diencephalon and midbrain. *Eurycea* and *Desmognathus* both have the relatively smallest forebrains, but whereas *Eurycea* has the relatively largest diencephalon and midbrain, these regions are relatively small in *Desmognathus*. Consequently, *Desmognathus* also has by far the relatively largest rhombencephalon of the series.

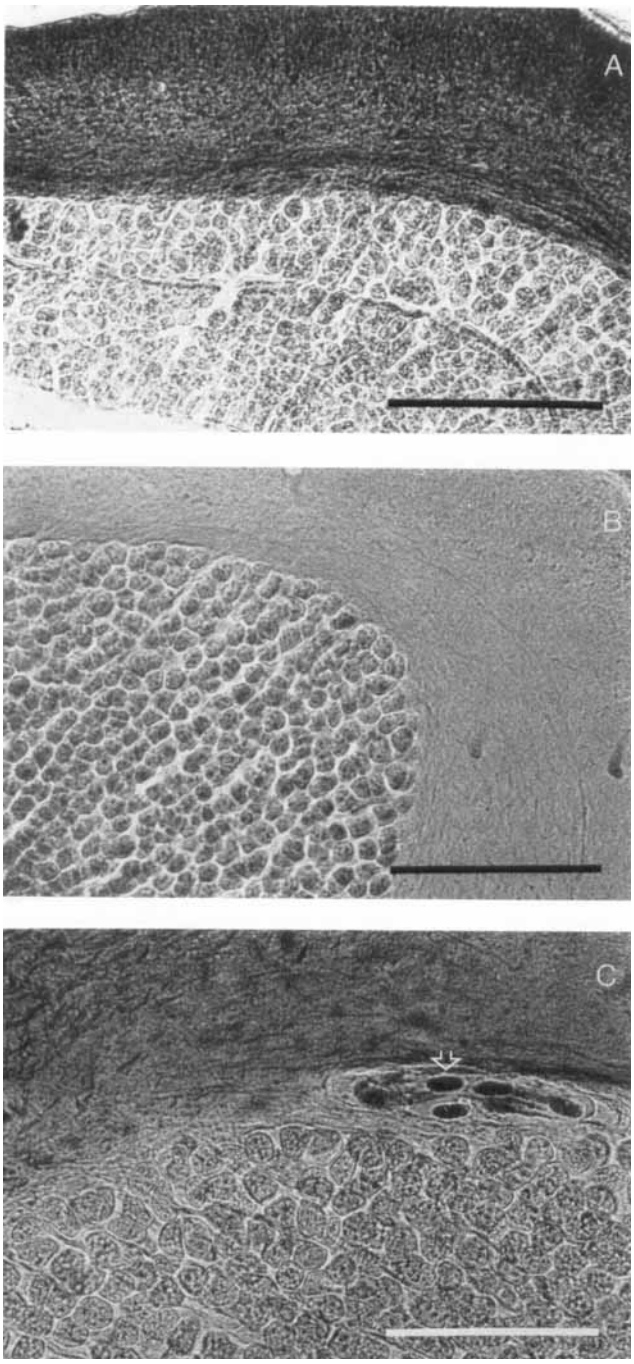

Figure 7. Photomicrographs of transverse sections (Palmgren stain) through the tectum mesencephali of A, *Desmognathus ochrophaeus*, B, *Thorius narisovalis* and C, *Hydromantes italicus*, showing parts of the fibre layer (above) and the cellular layer (below). Arrow in C points to a blood vessel with erythrocytes. Scale bar = 100 μm.

Cell diameters in the optic tectum and midbrain tegmentum

Diameters of nerve cell perikarya in the midbrain (optic tectum and tegmentum) vary interspecifically (Table 6; Fig. 7). The three non-bolitoglossine species—*Desmognathus* (Fig. 7A), *Eurycea* and *Plethodon*—have the smallest cells, whereas all four bolitoglossine species have significantly larger cells ($P<0.001$). Among bolitoglossines, cell size is generally proportional to body size; by far the largest cells are found in *Hydromantes* (Fig. 7C). There is no significant difference in cell diameters between the optic tectum and the tegmentum in any species. Cell diameters are on average 15% smaller in Palmgren preparations than in Golgi preparations; patterns of interspecific variation are the same in both types of material.

Ratio of grey matter to white matter in the diencephalon and midbrain

The relative proportions of grey and white matter (i.e. the periventricular layer containing perikarya, proximal dendritic shafts, and basal dendrites of neurons and glial elements *vs.* the superficial layer containing afferent fibres, neuronal dendrites, radial processes of glial ependymal cells, and efferent fibres) varies interspecifically (Table 7). The smallest relative amount of grey matter, and, therefore, the largest relative amount of white matter, is found in the three non-bolitoglossine species (*Plethodon, Desmognathus* and *Eurycea*) with small cells. In these species, the ratio of grey to white matter is about 1 : 2. The relative volume of grey matter is considerably larger in the four bolitoglossine species, which include both miniaturized salamanders with medium-sized cells (*Thorius, Batrachoseps*) and large salamanders with large cells (*Bolitoglossa, Hydromantes*).

Relative volume of grey matter in the tectum

Estimates of the volume of grey matter in the tectum relative to that in the tegmentum (and thus of the whole mesencephalon) are presented in Table 8. Values obtained from Palmgren material are higher than those from Golgi material in six of the seven genera. This may be due to greater shrinkage of white matter in Palmgren material. Nevertheless, patterns of interspecific variation are similar in the two preparations. The distribution of values derived from Palmgren material is U-shaped: high in both the smallest and largest species (i.e.

TABLE 7. Relative proportion of grey matter and white matter in the midbrain. $N = 3$ specimens per species and histological preparation

Genus	Grey matter			White matter		
	Palmgren	Golgi	Mean	Palmgren	Golgi	Mean
Thorius	42.4	41.6	42.0	57.6	58.4	58.0
Batrachoseps	47.1	41.6	44.4	52.9	58.4	55.6
Desmognathus	35.9	30.4	33.2	64.1	69.6	66.8
Eurycea	36.3	42.0	39.2	63.7	58.0	60.8
Plethodon	35.7	34.9	35.3	64.3	65.1	64.7
Bolitoglossa	45.0	37.0	41.0	55.0	63.0	59.0
Hydromantes	50.2	40.3	45.3	49.8	59.7	54.7

TABLE 8. Relative volume of grey matter in the tectum expressed as a percentage of grey matter in the whole mesencephalon. $N = 3$ specimens per species and histological material

Genus	Palmgren	Golgi	Mean
Thorius	84.4	69.1	76.8
Batrachoseps	68.8	60.0	64.4
Desmognathus	63.3	48.7	56.0
Eurycea	59.5	50.0	54.8
Plethodon	66.3	72.2	69.3
Bolitoglossa	70.9	55.6	63.3
Hydromantes	70.1	61.5	65.8

the four bolitoglossines), low in the medium-sized ones (the three non-bolitoglossines); the relative volume of tectal grey matter is highest in *Thorius* (84.4%). In the Golgi material, values for the non-bolitoglossines are again lower than those for the bolitoglossine species, except for *Plethodon* which has the highest value among the seven genera. The reasons for this discrepancy are unclear. Notwithstanding this exception, miniaturization *and* large cell size seem to be associated with an increase in relative volume of tectal grey matter.

Cell density in the diencephalon and midbrain

Cell density (i.e. the volume of perikarya relative to the total volume of grey matter in the tectum opticum, the midbrain tegmentum, and the whole midbrain) was determined from Palmgren and Golgi preparations (Table 9). Although the specific ordering of species differs between the two types of material, common patterns emerge. Cell density is lowest in the non-bolitoglossine species with small cells—viz. *Eurycea,* followed by *Desmognathus* and *Plethodon.* In contrast, all four bolitoglossine species have substantially higher cell densities ($P<0.001$). Thus, the two smallest and the two largest species have remarkably similar packing densities, In *Batrachoseps,* cell density is

TABLE 9. Nerve cell density (ratio of perikarya to periventricular grey matter, expressed as a percentage) in the tectum opticum (TO), the midbrain tegmentum (TG), and the whole midbrain (MB) weighted according to the ratio of tectum volume to tegmentum volume. Each value denotes the mean of three specimens per species and histological material

Genus	Palmgren			Golgi		
	TO	TG	MB	TO	TG	MB
Thorius	32.7	25.3	31.5	29.2	20.5	26.5
Batrachoseps	24.8	24.8	24.8	27.3	27.6	27.4
Desmognathus	17.3	16.7	17.0	18.0	14.7	17.1
Eurycea	16.2	15.8	16.0	17.1	11.5	14.3
Plethodon	16.8	16.4	16.7	23.2	17.4	20.2
Bolitoglossa	26.7	21.0	25.0	31.7	26.8	29.5
Hydromantes	29.6	26.4	28.6	38.0	24.4	32.7

approximately the same in the tectum opticum and the tegmentum. In all six remaining species, cell density is higher (sometimes considerably) in the tectum opticum.

Volume of the cellular layers and number of neurons in the diencephalon and midbrain

In both Palmgren and Golgi preparations, total volume of the diencephalon (minus hypothalamus) and the midbrain (tectum, praetectum and tegmentum) is largest in *Hydromantes* and then *Bolitoglossa;* it is smallest in *Thorius*, followed by *Desmognathus* and *Plethodon* (Table 10). There is a discrepancy concerning the rank order of *Batrachoseps* and *Eurycea,* but this may reflect the different sizes of specimens used for the two preparations.

The total number of neurons within the periventricular grey matter of regions containing the major visual centres (diencephalon plus mesencephalon) lie within a narrow range of 177 316–56 145 (ratio of 3.2 : 1) in Palmgren material and 196 352–59 621 (ratio of 3.3 : 1) in Golgi material (Table 11). In both preparations, *Hydromantes* has the most cells and *Thorius* and *Batrachoseps* the least, but there are differences in the rank order of the remaining species. The

TABLE 10. Total volume ($mm^3 \times 10^{-3}$) of the diencephalon (minus hypothalamus), the tectum, the praetectum, and the tegmentum in Palmgren and Golgi preparations. Values denote mean ± SE; $N = 3$

Tables	Palmgren	Golgi
Thorius	156 ± 2	270 ± 26
Batrachoseps	258 ± 24	477 ± 48
Desmognathus	179 ± 19	370 ± 24
Eurycea	295 ± 27	438 ± 11
Plethodon	246 ± 9	410 ± 48
Bolitoglossa	621 ± 68	931 ± 27
Hydromantes	1,180 ± 23	1,807 ± 77

TABLE 11. Total number of neurons in the (a) diencephalon (minus hypothalamus) and mesencephalon, and in the (b) tectum opticum. $N = 3$ specimens per species and histological preparation

Genus		Golgi	Palmgren	Mean
Thorius	a	59 621	56 145	57 883
	b	33 333	37 425	35 379
Batrachoseps	a	93 445	87 346	90 396
	b	36 567	43 290	39 929
Desmognathus	a	141 299	147 024	144 162
	b	52 296	57 383	54 839
Eurycea	a	140 027	122 203	131 155
	b	53 004	56 344	54 674
Plethodon	a	154 493	105 686	130 090
	b	53 853	50 544	52 199
Bolitoglossa	a	136 178	134 926	135 552
	b	54 114	59 434	56 774
Hydromantes	a	196 352	177 316	186 834
	b	90 058	84 373	87 216

fact that *Bolitoglossa*, despite its relatively large brain, ranks only third in the Palmgren material and fifth in the Golgi material is especially surprising.

The species are much more similar with respect to the number of neurons in the optic tectum (Table 11). Although values range from 90 058 to 33 333 in Golgi preparations, and from 84 373 to 37 425 in Palmgren material, cell number varies minimally among all species except *Hydromantes* (which accounts for both high values), despite huge differences in brain size.

In interpreting the above estimates of total numbers of neurons within the regions containing the major visual centres, it is important to remember that the periventricular grey matter of the thalamic and mesencephalic regions contains, in addition to visual neurons, a hitherto undescribed component of non-visual (e.g. somato-sensory) neurons, as well as non-neuronal glial elements (Naujoks-Manteuffel & Roth, 1989). Thus, the actual numbers of thalamic, tectal/pretectal, and tegmental neurons involved in visual and visuomotor functions may be even lower than those reported here.

DISCUSSION

Among amphibians, plethodontid salamanders are remarkable in many respects. All are lungless, and most lack aquatic larvae (Wake, 1966). Many species have a highly specialized, visually guided feeding mechanism that includes a high-speed, highly protrusible tongue (Lombard & Wake, 1976, 1977; Roth, 1976, 1987; Thexton, Wake & Wake, 1977). Many species have enormous genome and cell sizes (Sessions, 1984), while at the same time some species are extraordinarily small (Hanken, 1983, 1984, 1985). All or some of these features likely are interconnected in complex ways that influence both morphology and function (cf. Wake, 1982; Roth & Wake, 1985). Of special interest is the relation between genome/cell size and miniaturization, because these parameters directly affect morphology and function of those systems that guide behaviour—viz. sense organs and the nervous system.

First, we review the trends associated with miniaturization in plethodontid salamanders. Then, we incorporate the complicating effects introduced by interspecific variation in genome and cell size. Finally, we discuss our results with respect to the limits to visual function and design, particularly as they relate to miniaturization in plethodontid salamanders.

Compensations for miniaturization

Miniaturization has occurred independently several times among plethodontid salamanders. Miniaturized species include, for example, *Desmognathus wrighti, Batrachoseps attenuatus, Parvimolge townsendi,* and all members of the genus *Thorius*. In overall body size, *Thorius pennatulus* is among the smallest land vertebrates; in head, eye and brain size, it probably is the smallest one. Consequently, there is enormous disparity in head size between small and large species. The head volume of adult *Thorius*, for instance, is approximately only one-twenty-seventh that of *Hydromantes*, the largest species we have considered; *Batrachoseps*, while larger than *Thorius*, still has a head volume less than one-tenth that of *Hydromantes* (Table 2).

Effectiveness of the visual system (including the eyes and the visual and visuomotor centres in the brain) commonly is believed to depend on absolute cell mass, which in turn is a function of receptor and cell number and size. All the species dealt with herein rely heavily on vision (cf. Roth, 1987), but this is especially true for the four bolitoglossines which have a projectile tongue and show very precise and rapid feeding reactions (Roth, 1976; Thexton *et al.*, 1977). The much smaller cell mass of the visual system in miniaturized species might be expected to diminish or otherwise reduce the effectiveness of the visual guidance of behaviour in these forms. Several features of brain design, however, seem to compensate for the decrease in absolute brain and receptor size, thereby allowing even the smallest bolitoglossines to maintain visual function.

The most conspicuous compensatory feature involves negative allometry of the eye and the brain; both are relatively larger in smaller salamanders. For example, the ratio of head volumes of *Hydromantes* and *Thorius* is about 27 : 1. The ratio of eye volumes, however, is approximately 18 : 1, and the ratio of brain volumes is about 8 : 1. Thus, in *Thorius* the eyes and brain each occupy 10–12% of head volume, instead of the 4–7% occupied in the larger species. Newly hatched *Hydromantes* have a relative brain volume of about 12%, equal to adult *Thorius*. In contrast, relative brain volumes of newly hatched *Batrachoseps* and *Bolitoglossa* are 16.5 and 20%, respectively. Moreover, they possess 60–75% of the total adult number of tectal neurons; this is associated with a high (as much as 48%) density of tectal neurons in hatchlings (Roth *et al.*, 1988).

Several internal features of the eye also likely compensate for its tiny absolute size in miniaturized taxa. These include a relatively larger retinal volume (seemingly at the expense of the size of the lens, which is reduced), a relatively larger layer of retinal ganglion cells, a higher proportion of small-sized cones in comparison to large-sized rods, and denser packing of receptors and retinal ganglion cells (Linke *et al.*, 1986). Each of these features maximizes the number of optic fibres (as processes of retinal ganglion cells) and thus enhances visual acuity necessary for feeding.

Within the brain, five morphological features seem to compensate for its small absolute size in miniaturized taxa. First, ventricular volume is relatively small. Second, the volume of those brain regions that contain the major visual and visuomotor centres—viz. the thalamus, the praetectum, and the whole midbrain including the optic tectum and the different tegmental regions—is relatively large. Within the thalamus, the anterior and posterior pars dorsalis and pars ventralis thalami, the nucleus Bellonci, and at least part of the pars intercalaris thalami are involved in processing primary visual information (e.g. barrier detection). The praetectum and the rostral tegmentum include the nuclei praetectalis, Darkschewitsch, fasciculi longitudinalis medialis, tuberculi posterioris, and ventralis tegmenti, along with the nuclei of the oculomotor and trochlear nerves. All of these nuclei are involved in visual and visuomotor functions related to barrier detection, optomotor behaviour, and figure-ground discrimination (cf. Roth, 1987). The tectum mesencephali is the main visual centre that, together with the retina, contains the mechanisms necessary for visual orientation and visual acuity. The size of the tectum and the number of neurons it contains determine the precision of visually oriented behaviour and object recognition—factors that are important in feeding and enemy avoidance. The tegmentum mesencephali also contains the nucleus isthmi, which is

connected exclusively to the tectum and has only visual functions (cf. Roth, 1987). The predominance of visual and visuomotor centres within these brain areas varies interspecifically. Among the species studied here, visual centres and functions occupy roughly 66% of the diencephalic areas measured, 100% of the praetectum, 80–90% of the tectum, and 66% of the tegmentum mesencephali (Naujoks-Manteuffel & Roth, unpublished data).

A third feature that we interpret as compensation for miniaturization is the increased volume of grey matter containing primarily neuronal perikarya relative to the volume of white matter containing neuronal dendrites and afferent and efferent fibres. An increase in the proportion of grey matter means that there is relatively more space available for neurons. Interestingly, a large relative volume of grey matter is generally characteristic of all bolitoglossine salamanders, which also have large cells compared to non-bolitoglossine species.

A fourth feature is the larger volume of tectal grey matter relative to that of tegmental grey matter. The relative difference in volume is exceptionally marked in *Thorius*, in which the tectum is 5.4 times as large as the tegmentum. The significance of this difference with regard to the visuomotor function of the tegmentum is unknown.

Fifth, there is increased cell density in the packing of perikarya within the grey matter. However, equally high densities occur in both miniaturized salamanders and large salamanders with extremely large cells.

Combined, these morphological features that we interpret as compensating for small size result in the remarkable cranial configuration in miniatures, as exemplified by *Thorius*. The head of *Thorius* is one-twenty-seventh and the brain is one-ninth the size of the same structures in the much larger *Hydromantes*. Yet, *Thorius* has only slightly less than one-third as many thalamic, pretectal, and mesencephalic neurons than does *Hydromantes*. In short, the features are effective in maintaining a certain minimum number of neurons involved in visual and visuomotor processing.

Effects of genome and cell size

In amphibians, indeed in animals generally, cell size is positively correlated with genome size (see reviews by Szarski, 1983; Cavalier-Smith, 1982; Horner & MacGregor, 1983). This relationship also holds for the plethodontid salamanders we studied; species having larger genomes have larger cells, including photoreceptors and neurons (Fig. 8). The extremes are represented by *Desmognathus ochrophaeus*, with a mean nerve-cell diameter of 8.9 μm, and *Hydromantes*, with a diameter of 16.4 μm. Corresponding mean cell volumes are 367 μm^3 and 2310 μm^3, respectively. Thus, in *Hydromantes*, the average volume of a single neuron is about six times that in *Desmognathus*. There is also a phylogenetic component to genome-cell size variation; the three non-bolitoglossine species have the smallest genomes and cells, whereas all four bolitoglossine species have larger values.

Large cells, as a consequence of large genome size, may introduce problems for the eyes and brains of relatively large salamanders that are remarkably similar to those faced by miniaturized species. For example, for a given eye and brain size, and in the absence of compensatory processes, increased cell size would be

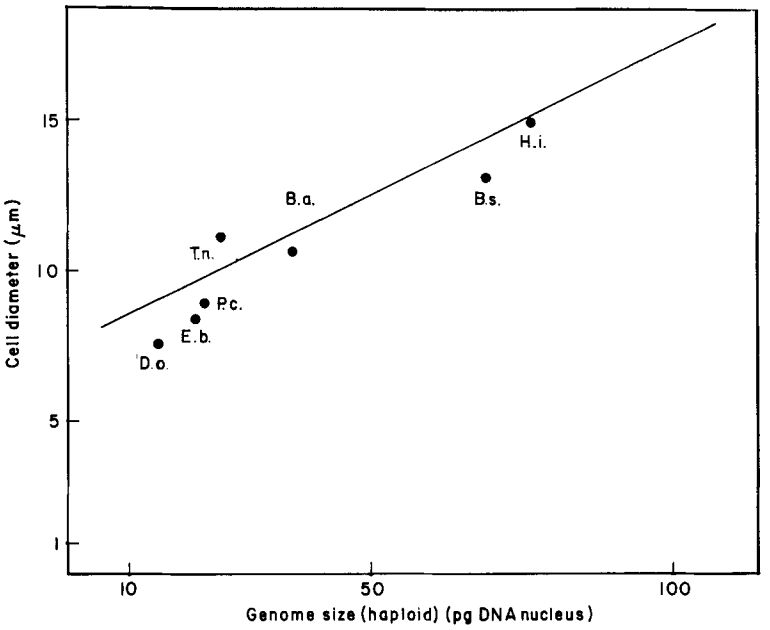

Figure 8. Diameter of tectal neurons (Palmgren material) as a function of haploid genome size (pg DNA/nucleus) in plethodontid salamanders. Species are listed in the legend to Figure 4.

associated with a tremendous decrease in the number of receptors and neurons. These problems are potentially the most severe in two species we have considered: *Hydromantes*, which has both the largest genome of any terrestrial vertebrate and the largest cells, including neurons, at least among terrestrial amphibians; and *Bolitoglossa*, which has the second largest known genome and cell sizes in terrestrial amphibians. Thus, it is not surprising to see features of visuomotor design and packaging in *Hydromantes* and *Bolitoglossa*, the two largest species in the series, that mirror those in *Thorius*, the smallest species.

First, both *Hydromantes* and *Bolitoglossa* have much larger eyes than would be expected based on simple allometry with respect to head size (Fig. 6). We suggest that if their eyes were substantially smaller, as they would be if eye size scaled to head and body size the same way it does in intermediate-sized species, and if neuron and receptor sizes were unchanged, then the number of neurons and receptors likely would lie below the minimum necessary for proper function—e.g. for visual acuity in these tongue-projecting salamanders. Second, the ratio of grey to white matter in *Hydromantes* is even larger (in Palmgren material) than in the two miniaturized species, *Thorius* and *Batrachoseps* (Table 7). Finally, in *Hydromantes*, cell density (i.e. the ratio of nerve cell perikarya to periventricular grey matter within the brain areas containing visual centres) is nearly equal to (Palmgren material) or larger than (Golgi material) that in *Thorius*.

Thorius represents an exception to the otherwise pervasive trend towards genome-size increase that is characteristic of bolitoglossine salamanders. Indeed, genome size in *Thorius* is 27% less than the estimated ancestral value (34.5 pg) for the tribe Bolitoglossini (Sessions & Larson, 1987). At present, however, we

are unable to conclude whether this reversal in genome size preceded miniaturization, thereby satisfying a necessary (possibly developmental) precondition for body size decrease, or if it accompanied miniaturization as an important adaptive and compensatory feature.

Limits to function

Despite the remarkable morphological features that seem to compensate for both decrease in body size and increase in cell size in plethodontid salamanders, all species we have studied have very low numbers of photoreceptors, retinal ganglion cells, and neurons within the brain regions containing the main visual and visuomotor centres. The number of retinal ganglion cells in plethodontids ranges from about 52 000 (*Eurycea*) to 26 500 (*Batrachoseps*) (Linke *et al.*, 1986); these values are significantly lower than those reported for salamandrids (cf. Roth, 1987) and only about one-tenth those in anurans (Maturana, 1959). Unfortunately, there is no precise estimate of the number of neurons in the central visual regions of any other amphibian, and only a rough estimate (based on Golgi material) of 800 000 tectal neurons in *Rana esculenta* (Székely & Lázár, 1976). If this number is accurate, then plethodontid salamanders not only have ten-times fewer retinal ganglion cells and optic nerve fibres, but also twenty-times fewer tectal neurons.

Nevertheless, the visual system of plethodontid salamanders is extremely efficient, at least with regard to visual acuity, depth perception, and visuomotor coordination. Visual acuity estimated from eye morphology, as well as behavioural and electrophysiological data, indicate that even small plethodontids are able to detect small prey such as collembolans or fruit flies at distances of 30–50 cm (Linke *et al.*, 1986; Roth, 1987). Indeed, most tongue-projecting salamanders typically prey on such tiny food, and at least some bolitoglossines are able to catch flying insects (Wake, personal communication). In addition, tongue-projecting Bolitoglossini have substantial, direct binocular retinal projections to thalamic, pretectal and tectal visual centres and an organization of visual neuropils that is unusual among lower vertebrates (Rettig & Roth, 1986).

We interpret the trends in neural and ocular design described above as compensatory mechanisms for the morphological and functional demands imposed by both miniaturization and increased genome and cell size, whether individually or in combination. Specifically, the changes in brain and eye structure and organization maintain a high level of visual performance during feeding. We further suggest that the ultimate explanation for the evolution of miniaturized species and increased genome size lies far removed from neural physiology and, indeed, visual function generally (cf. Szarski, 1983). For miniaturization, a more likely explanation may lie in the ecological advantages conferred by small size. For increased genome size, explanations range from 'selfish' DNA (Orgel & Crick, 1980; Doolittle & Sapienza, 1980) to an adaptive 'frugal' strategy leading to economics of energy and material, because of a strong decrease in the rate of cell metabolism (Szarski, 1983).

Nevertheless, visual system design may represent a primary limit to both body-size decrease and genome- and cell-size increase in plethodontid salamanders. Indeed, in view of the numerous, extreme modifications of neural

and ocular design that we have described above and in earlier studies, it is difficult to imagine that further reduction in body size could be achieved in salamanders with medium-sized cells (e.g. *Thorius*) or those with large cells (e.g. *Hydromantes*). Instead, further reduction of body size in these lineages, without an accompanying reduction in genome and cell size, may be possible only by impairment or even loss of visual function, and an associated, drastic shift in trophic specialization.

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ABBREVIATIONS USED IN THE FIGURES

ca	commissura anterior	MO	medulla oblongata
Cb	cerebellum	MS	medulla spinalis
ch	commissura hippocampi	nB	nucleus Bellonci
ch.o	chiasma opticum	nBON	nucleus neuropili optici basalis
cp	commissura posterior	nD	nucleus Darkschewitsch
c.po	commissura postoptica	nDTa	nucleus dorsalis tegmenti anterior
D/DI	diencephalon	nDTp	nucleus dorsalis tegmenti posterior
ETh	eminentia thalami	nflm	nucleus fasciculi longitudinalis medialis
HD	habenula dorsalis	nPOa	nucleus praeopticus anterior
HV	habenula ventralis	nPOp	nucleus praeopticus posterior
Hy	hypothalamus	nPT	nucleus praetectalis
M/MES	mesencephalon	ntOH	nucleus tractus olfacto-habenularis

nTP	nucleus tuberculi posterioris
nVTa	nucleus ventralis tegmenti anterior
nVTp	nucleus ventralis tegmenti posterior
nIII	nucleus nervi oculomotorii
nIV	nucleus nervi trochlearis
pDTha	pars dorsalis thalami anterior
pDThp	pars dorsalis thalami posterior
PITh	pars intercalaris thalami
pVTha	pars ventralis thalami anterior
pVThp	pars ventralis thalami posterior
T/TEL	telencephalon
Tg	tegmentum mesencephali
Th	thalamus
To	tectum opticum
I	nervus olfactorius
II	nervus opticus
III	nervus oculomotorius
V	nervus trigeminus
VII	nervus facialis
VIII	nervus stato-acusticus
IX	nervus glossopharyngeus
X	nervus vagus
XI	nervus accessorius