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Distribution of GABA, Glycine, and Glutamate in Neurons of the Medulla Oblongata and Their Projections to the Midbrain Tectum in Plethodontid Salamanders

SANDRA LANDWEHR AND URSULA DICKE*
Brain Research Institute, University of Bremen, D-28334 Bremen, Germany

ABSTRACT

In the medulla oblongata of plethodontid salamanders, GABA-, glycine-, and glutamate-like immunoreactivity (ir) of neurons was studied. Combined tracing and immunohistochemical experiments were performed to analyze the transmitter content of medullary nuclei with reciprocal connections with the tectum mesencephali. The distribution of transmitters differed significantly between rostral and caudal medulla; dual or triple localization of transmitters was present in somata throughout the rostrocaudal extent of the medulla. Regarding the rostral medulla, the largest number of GABA- and gly-ir neurons was found in the medial zone. Neurons of the nucleus reticularis medius (NRM) retrogradely labeled by tracer application into the tectum revealed predominantly gly-ir, often colocalized with glu-ir. The NRM appears to be homologous to the mammalian gigantocellular reticular nucleus, and its glycinergic projection is most likely part of a negative feedback loop between medulla and tectum. Neurons of the dorsal and vestibular nucleus projecting to the tectum were glu-ir and often revealed additional GABA- and/or gly-ir in the vestibular nucleus. Regarding the caudal medulla, the highest density of GABA- and gly-ir cells was found in the lateral zone. Differences in the neurochemistry of the rostral versus caudal medulla appear to result from the transmitter content of projection nuclei in the rostral medulla and support the idea that the rostral medulla is involved in tecto-reticular interaction. Our results likewise underline the role of the NRM in visual object selection and orientation as suggested by behavioral studies and recordings from tectal neurons. J. Comp. Neurol. 490:145–162, 2005.

Indexing terms: transmitter colocalization; brainstem; amphibians; reticular formation; nucleus reticularis medius; Mauthner neuron

The medulla oblongata is an anatomically and functionally heterogeneous part of the vertebrate brain. It is concerned with the control of movements and of vital body functions such as respiration and blood circulation and contains primary and secondary relay stations for somato- and viscerosensory information and reticular nuclei involved in the control of vigilance and attention. Furthermore, it is the convergence zone of descending pathways from all parts of the brain (for a comparative overview, see Butler and Hodos, 1996; Nieuwenhuys et al., 1998). In the amphibian medulla oblongata, components of the auditory, vestibular, and somatosensory system as well as the anatomy and distribution of the cranial motor nuclei V–XII have been studied (Roth et al., 1988; Székely and Matesz, 1993; Muñoz et al., 1994; Strake et al., 1994; Dicke and Mühlenbrock-Lenter, 1998; Straka and Dieringer, 2000). Likewise, numerous ascending and descending connections between the medulla and other

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*Correspondence to: Ursula Dicke, Brain Research Institute, University of Bremen, D-28334 Bremen, Germany. E-mail: dicke@uni-bremen.de

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brain areas have been documented by tracing studies (ten Donkelaar et al., 1981; ten Donkelaar, 1982; Naujoks-Manteuffel and Manteuffel, 1988; Dicke et al., 1998). In plethodontid salamanders the tectum mesencephali as the main visual center exhibits strong reciprocal connections with nuclei in the rostral medulla oblongata, viz., the nucleus reticularis medius, the nucleus vestibularis, and the nucleus dorsalis (Dicke and Mühlenbrock-Lenter, 1998; Dicke, 1999; Roth et al., 1999). Studies on orienting behavior as well as electrophysiological recordings from tectal neurons during visual object selection in the salamander *Plethodon* reveal modulatory effects of the visual surround on tectal information processing (Schuelert and Dicke, 2002, 2005). These modulations have been assumed to originate from an interaction between the tectum and more caudally situated brainstem regions such as the reticular nuclei. Therefore, information on the transmitter specificity of neurons in the medulla oblongata and especially in ascending projection nuclei could further elucidate the functional relationship between tectum and medulla. In amphibians, a number of brainstem nuclei have been characterized by their content of neurotransmitters and -peptides, i.e., the serotonergic raphe nuclei (Adli et al., 1999; Dicke et al., 1997; Stuesse et al., 2001), the noradrenergic locus coeruleus (Marín et al., 1996), and the cholinergic cranial motor nuclei and laterodorsal tegmental nucleus (Marín et al., 1997). Among amino acid transmitters, glycine and GABA have been shown to exert modulatory effects on tectal processing (Sivilotti and Nistri, 1986; Kahl, 1999), but their precise localization in the medulla oblongata has not been demonstrated, with one exception mentioned below. While in the mammalian medulla oblongata the distribution of amino acid transmitters has been studied in greater detail revealing, for example, a characteristic distribution of GABA, glycine, or glutamate in different functional systems (Adams and Mugnaini, 1990; Jones, 1995; Li et al., 1996, 1997; Kobayashi et al., 1997; Suzuki et al., 1997; Rampon et al., 1999), in amphibians a similar study was carried out only with regard to the vestibular nuclei of *Rana* (Reichenberger et al., 1997). In the present study we investigated the transmitter distribution of GABA, glycine, and glutamate and their colocalization in the medulla oblongata of two salamander species, *Plethodon shermani* (former *Plethodon jordani*) and *Hydromantes genei*. In nuclei of the rostral medulla with ascending projections to the tectum, combined biocytin tracing and immunohistochemical experiments were performed to determine the transmitters involved in reticulo-tectal interaction. This study aims at the elucidation of the integrative role of the amphibian medulla oblongata in the context of visuomotor behavior and specifically of the interaction between medulla and tectum.

MATERIALS AND METHODS

Specimens of *Plethodon shermani* (sensu Highton and Peabody, 2000; n = 27; 5 females; 22 males; snout-vent length (SVL) = 52–56 mm) were collected from wild populations in the vicinity of Highlands Biological Station, North Carolina, and specimens of *Hydromantes genei* (n = 6; 4 females, 2 males; SVL = 55–60 mm) were collected in the vicinity of Domusnuovas, Sardinia, Italy, and kindly provided by the Zoological Institute of the University of Cagliari. Animals were housed communally in terraria at 18°C on a light/dark cycle of 12/12 hours and fed crickets. The experiments were approved by the veterinary office of the state of Bremen, Germany, and conform to NIH guidelines. The distribution of GABA-like-immunoreactivity (GABA-ir) in the medulla oblongata was studied in seven and of glycine-like immunoreactivity (gly-ir) in eight individuals of *Plethodon*. In *Hydromantes* the distribution of GABA-ir and gly-ir was studied in two animals each. The colocalization of GABA-ir, gly-ir, and glutamate-like immunoreactivity (glu-ir) was investigated in eight *Plethodon* and two *Hydromantes*. In four *Plethodon* these experiments were combined with biocytin tracing. Neurons in projection nuclei in the rostral medulla, viz., the nucleus dorsalis (ND), the nucleus vestibularis (NV), and the nucleus reticularis medius (NRM), were retrogradely labeled by tracer application in the tectum. Animals were anesthetized in a solution containing 2% 3-aminobenzoic acid ethyl ester (MS 222, Sigma Chemical, St. Louis, MO); Xylocain (Astra Chemicals, Wedel, Germany) was used as a local anesthetic in addition. After opening the dorsal skull the dura mater and the leptomeninges were removed above the tectum. The fiber layers of the lateral tectum were lesioned with the sharpened tip of a glass electrode and crystals of biocytin (Sigma) were applied uni- or bilaterally. The lesions were restricted to the mid-tectum and extended roughly 400 μm rostrocaudally. Animals survived for 22–24 hours. For immunohistochemistry, animals were reanesthetized and transcardially perfused with Ca_2^+-free Ringer's solution, followed by a fixative containing 3% paraformaldehyde (PFA) and 2% glutaraldehyde (GA) in the case of localization of GABA-ir and colocalization of transmitters, and 1% PFA and 2% GA for the detection of gly-ir. After removal, brains were postfixed overnight at 4°C. Transverse sections of brains were made on a vibratome (Leica, Germany). Sections were transferred to chromalum-gelatin-coated slides. For colocalization of transmitters including those with combination of biocytin detection, the procedure largely follows Reichenberger et al. (1997). Slices of 100 μm were cut on a vibratome, dehydrated, and transferred to epoxy resin (Epon 812, Serva, Germany). Selected Epon-embedded slices from the rostral medulla oblongata and from the level of the entrance of the Xth and XIth cranial nerve were cut on an ultramicrotome (Leica). A series of three or four semithin sections was mounted onto consecutive chromalum-gelatin-coated slides and etched for removal of epon. Sections on one slide were stained either for GABA, glycine, glutamate, or biocytin, with a preincubation in sodium borohydride (0.1 M) for GABA and glycine. The thickness of sections, the dilution of antibodies and sera, and the buffers used are given in Table 1. After incubation in normal serum (goat and horse serum; NGS and NHS, respectively; Vector Kit, Burlingame, CA; swine and rabbit serum; NSS and NRS, respectively; DAKO, Germany) for 1–2 hours at room temperature, a polyclonal antibody against GABA (Sigma, affinity-purified) or glycine (Chemicon, Germany), or a monoclonal antibody against glutamate (Sigma) was applied to the sections. Sections were incubated in primary antibodies overnight at room temperature. After rinsing in buffer, sections were incubated in biotinylated IgG (goat antirabbit or horse antimouse IgG, Vector Kit) or swine antirabbit or rabbit antimouse IgG (DAKO). For the experiments using immunohistochemistry only, the avidin-biotin method (ABC; Vector Kit) was applied (Hsu et al., 1981), while the

TABLE 1. List of Antibodies, Dilutions, and Treatment

Type of experiment	Sections	Blocking system	Primary antibodies	Secondary antibodies	Detection system
Immunohistochemical localization	Vibratome 25 μm	NGS; Rabbit- Kit, 1: 600 in Tris-MBS	Rabbit anti-GABA; polyclonal, 1:2,000 in Tris-MBS Rabbit anti-glycine; polyclonal, 1:250 in Tris-MBS	Goat antirabbit-IgG; biotinylated, Rabbit-Kit, 1:200 in TBS	ABC; Rabbit-Kit, 1:100 in PBS
Colocalization	Ultramicrotome 900 nm	NGS; Rabbit-Kit, 1:600 in PBS	Rabbit anti-GABA; polyclonal, 1:2,000 in PBS Rabbit anti-glycine; polyclonal, 1:250 in PBS	Goat antirabbit-IgG; biotinylated, Rabbit-Kit, 1:200 in PBS	ABC; Rabbit-Kit, 1:100 in PBS
		NHS; Mouse-Kit, 1:600 in PBS	Mouse anti-glutamate; monoclonal, 1:10,000 in PBS	Horse antimouse-IgG; biotinylated, Mouse-Kit, 1:200 in PBS	ABC; Mouse-Kit, 1:100 in PBS
Combined tracing-immunohistochemical colocalization	Ultramicrotome 900 nm	NSS; 1:600 in PBS	Rabbit anti-GABA; polyclonal, 1:2,000 in PBS Rabbit anti-glycine; polyclonal, 1:250 in PBS	Swine antirabbit-IgG; 1:40 in PBS	PAP-Rabbit; 1: 300 in PBS
		NRS; 1:600 in PBS	Mouse anti-glutamate; monoclonal, 1:10,000 in PBS	Rabbit antimouse-IgG; 1:40 in PBS	PAP-Mouse; 1: 300 in PBS

Tris-MBS, TRIS-sodium metabisulfite buffer, 0.05 M, pH 7.5; TBS, Tris base with sodium chloride, 0.05 M, pH 7.6; PBS, phosphate buffer with sodium chloride, 0.05 M, pH 7.2. For the rest of abbreviations, see text.

peroxidase-antiperoxidase method (PAP; DAKO) was used in the case of transmitter detection when combined with detection of biocytin (Sternberger et al., 1970). In the latter experiments, sections for biocytin detection were blocked by a 30% solution of H_2O_2 prior to incubation in the avidin-biotin-HRP-complex (Vector Kit). Diaminobenzidine was used as chromogen and was heavy-metal-intensified with $NiSO_4$ and $CoCl_2$ (Adams, 1981). The sections were dehydrated and coverslipped with Eukitt. Controls were performed on some sections in each immunohistochemical experiment by omitting the primary antibody and replacing it with the incubation buffer. Preadsorption tests and controls of crossreactivity were performed on brain sections of *Plethodon* (n = 4) by using amino acid-glutaraldehyde complexes following Storm-Mathisen and Ottersen (1990), and Spirou and Berrebi (1997). Briefly, GABA, glycine, or glutamate were conjugated to GA (concentration of transmitter:GA = 1:2) and unconjugated GA was reduced with ethanolamine hydrochloride (1 M). The concentration of each amino acid transmitter in the complex was 10 times higher than that of the respective antiserum. Each antiserum was then preadsorbed overnight with the complex of its respective transmitter and the antisera were incubated with the complex of other transmitters for tests of crossreactivity. Sections of the medulla oblongata were incubated with the different complex solutions and stained following the procedure described above. In the control experiments neither were the adsorption controls specifically stained, nor did the polyclonal antisera against GABA and glycine and the monoclonal antiserum against glutamate reveal crossreactivity (Fig. 1). Analysis of sections was performed using a light microscope (AxioCam HR; Zeiss, Oberkochen, Germany). The diameter of immunopositive and nonimmunoreactive somata were measured and the mean was calculated. Images of sections were read into the computer. A labeled soma was defined as immunopositive when its gray tone was at least 25% above that of immunonegative somata. This discrimination was carried out using Photo-Paint 10 (Corel, Ottawa, Canada). For quantitative analysis, immunoreactive somata were counted in one-half of all sections of the medulla oblongata from the rostral pole to the obex in all individuals (*Plethodon*, n = 8; *Hydromantes*, n = 4), while the total number of somata was determined on every tenth section. The interzonal and intrazonal frequency of labeled somata within the longitudinal zones were calculated. The interzonal frequency indicates the ratio between the number of immunoreactive somata in one longitudinal zone and the total number of somata in one-half of the medulla oblongata, whereas the intrazonal frequency specifies the ratio of labeled cells in one longitudinal zone and the total number of cells in this zone. For quantitative analysis of colocalization of transmitters in consecutive sections stained for GABA, glycine, glutamate, or biocytin-labeled somata, several selected sets of consecutive sections were investigated in the rostral medulla from the level of the Vth to the IXth cranial nerves and caudally at the level of the Xth to the XIth cranial nerves in each individual (*Plethodon*: transmitters n = 6, including biocytin tracing experiments; *Hydromantes*: transmitters n = 2). The inter- and intrazonal frequencies and the colocalization of transmitters were determined and transmitters were localized in biocytin-labeled somata. Images of semithin sections were edited in a computer program (CorelDraw 10.0), and immunopositive and biocytin-labeled somata were color-coded, projected on top of each other, and counted. Statistical analysis of differences in zones are based on the number of labeled versus unlabeled somata of the rostral and caudal medulla. Sizes of the longitudinal zones between individuals and the interzonal and intrazonal frequencies of somata in the rostral and caudal medulla were compared using univariable ANOVA, post-hoc Tukey test, and Student's *t*-test for unpaired sample survey (SPSS 12.0, SPSS, Chicago, IL). Variances of cell numbers across individuals are given by the standard error. Diagrams were made using computer programs (CorelDraw 10.0, Microsoft Excel 2000) and micrographs of labeled neurons were scanned with a digital camera (AxioCam HR) at a resolution of 3900 × 3090 pixels.

RESULTS

The anatomy of the medulla and immunohistochemical data of the neurotransmitters GABA, glycine, and glutamate in the medulla oblongata are very similar in *Pleth-*

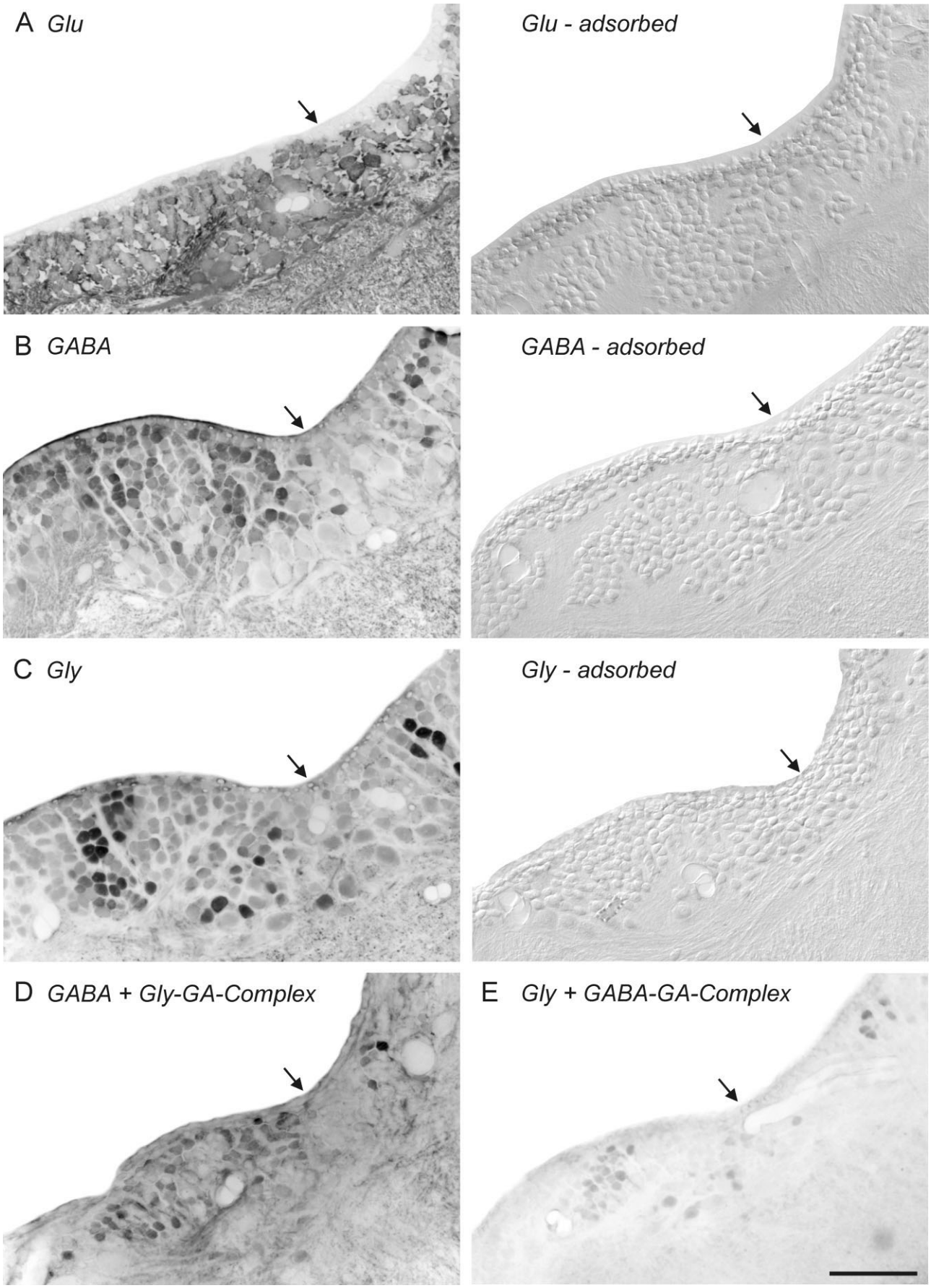


Fig. 1. Photomicrographs of sections through the same region of the rostral medulla showing the specificity of antiserum against glutamate, GABA, and glycine in the nonadsorbed (**A–C,** left panel) and adsorbed (A–C, right panel) conditions. **D:** Staining for GABA is cross-adsorbed against glycine and yields the same labeling pattern as in the nonadsorbed condition. **E:** Staining for glycine cross-adsorbed against GABA that results in the same labeling pattern as in the nonadsorbed condition. The arrows point to the sulcus limitans of His. Medial is to the left, dorsal is to the top. Scale bar = 100 μm in E (applies to A–E).

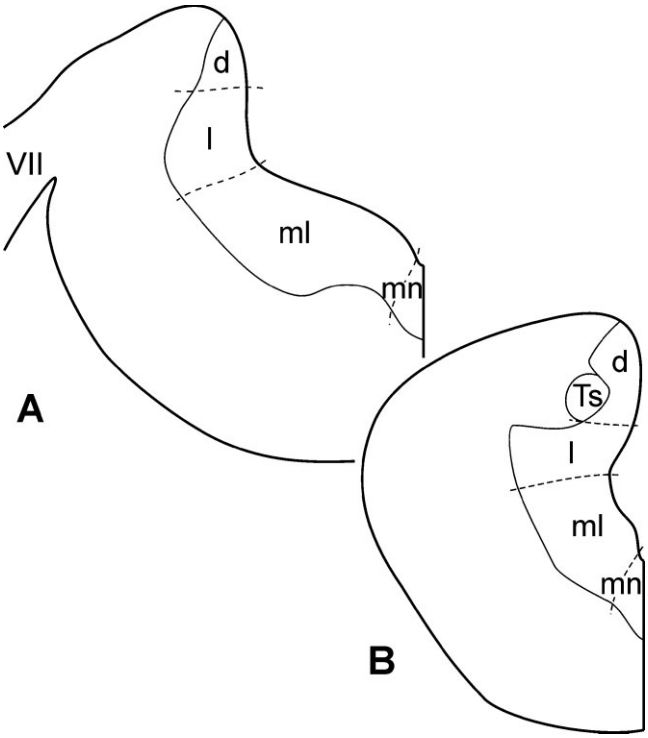


Fig. 2. Schematic diagram of transverse sections of one hemisphere of the medulla oblongata indicating the division into longitudinal zones. **A:** Rostral medulla at the level of the entrance of the VIIth cranial nerve (VII). **B:** Caudal medulla at the level between the entrances of the Xth and XIth nerve. mn, median; ml, medial; l, lateral; d, dorsal; Ts, solitary tract.

odon shermani and *Hydromantes genei*, although these two species belong to different tribes of the subfamily Plethodontinae (family Plethodontidae). The results of the anatomical and immunohistochemical experiments and statistical data will be presented only for *P. shermani*; only differences between the species are mentioned.

Size of longitudinal zones

In both species studied as well as in salamanders in general the medulla oblongata can be divided into four longitudinal zones, a median zone, a medial, a lateral, and a dorsal one. The median zone represents a narrow band characterized by the serotonergic raphe nuclei (Dicke et al., 1997); the medial zone is separated by the sulcus limitans of His from the lateral zone, which in turn is separated from the dorsal zone by the fasciculus solitarius (Fig. 2). The rostral medulla extends from the level of the entrance of the Vth to that of the IXth cranial nerve, and the caudal medulla from there to the obex. The gray matter of the medulla oblongata consists of a thick periventricular layer; few somata are found in a migrated position inside the white matter. In both species the median zone is small and comprises about 5% of total somata in the periventricular layer. The adjoining medial zone is the largest zone, comprising 60% of all somata in the cellular layer in the rostral and 45% in the caudal medulla oblongata. The size of the cellular layer in the lateral zone increases from 25% of all somata in the rostral to 30% in the caudal medulla. The periventricular layer in the dorsal zone comprises up to 10% of the cellular layer in the rostral and up to 20% in the caudal medulla. Statistical analysis revealed no significant differences in the sizes of the four longitudinal zones when individuals were compared.

Distribution of GABA- and glycine-like-immunoreactivity

The rostrocaudal length of the medulla oblongata ranged from 1,600–2,000 μm in *Plethodon* and from 2,000–2,600 μm in *Hydromantes*. In the transverse plane the average total number of neurons in the rostral medulla was 505 (standard error (SE) ± 35) in *Plethodon* and 591 (SE ± 31) in *Hydromantes*, and in the caudal medulla 420 (SE ± 21) and 416 (SE ± 11), respectively. The average total number of cells in one-half of the entire medulla was 28,700 (SE ± 3,200) in *Plethodon* and 39,600 (SE ± 2,400) in *Hydromantes*. In *Hydromantes* the entire medulla oblongata contains roughly 30% more neurons compared to *Plethodon*. The average total number of GABA-ir somata in the medulla oblongata was 23% (SE ± 3.59) of the total number of neurons in the rostral medulla oblongata of *Plethodon* and 25% (SE ± 4.33) of *Hydromantes*, and 22% (SE ± 2.51) in the caudal medulla oblongata of *Plethodon* and 25% (SE ± 2.45) of *Hydromantes*. Gly-ir somata represented 11% (SE ± 1.01) in the rostral medulla oblongata of *Plethodon* and 14% (SE ± 1.16) of *Hydromantes* of the total number of cells, and about 19% (SE ± 3.06) in the caudal medulla oblongata of *Plethodon* and 17% (SE ± 5.09) of *Hydromantes*.

The vast majority of immunopositive somata were located within the periventricular gray matter, but a few were found in a migrated position throughout the rostrocaudal extent of the medulla. The maximum number of migrated GABA-ir or gly-ir somata in one individual was 76 in *Plethodon* and 17 in *Hydromantes*. This difference corresponds to the generally larger number of migrated neurons in *Plethodon* compared to *Hydromantes*. Rostrocaudally, the distribution of immunolabeled somata varied in the different zones, but was similar in *Hydromantes* and *Plethodon*. Thus, the data presented in the following refer to both species, if not mentioned otherwise.

The frequency of the total number of GABA-ir and gly-ir neurons was approximately constant along the rostrocaudal extent of the medulla oblongata, but differed largely for the two transmitters when their interzonal frequency, i.e., the ratio between the number of immunoreactive somata in one longitudinal zone and total number of somata in one-half of the medulla oblongata, was taken into account (Fig. 3A,C). In the rostral medulla, the medial zone always contained the largest number of immunolabeled somata: on average 14% (SE ± 0.58) for GABA-ir and 9% (SE ± 0.24) for gly-ir somata in *Plethodon*. Each of the other zones contained less than 5% GABA-ir and gly-ir cells. In the caudal medulla, the medial, lateral, and dorsal zone contained roughly equal numbers of immunolabeled GABA-ir and gly-ir somata, i.e., 2–10% of total cell number, while in the median zone less than 1% labeled cells were included. When the intrazonal frequency of immunolabeled somata, i.e., the ratio of labeled cells and total number of cells in one longitudinal zone, is considered, GABA-ir and gly-ir somata are more evenly distributed (Fig. 3B,D). In the rostral medulla oblongata the medial zone still contains the largest number of immuno-

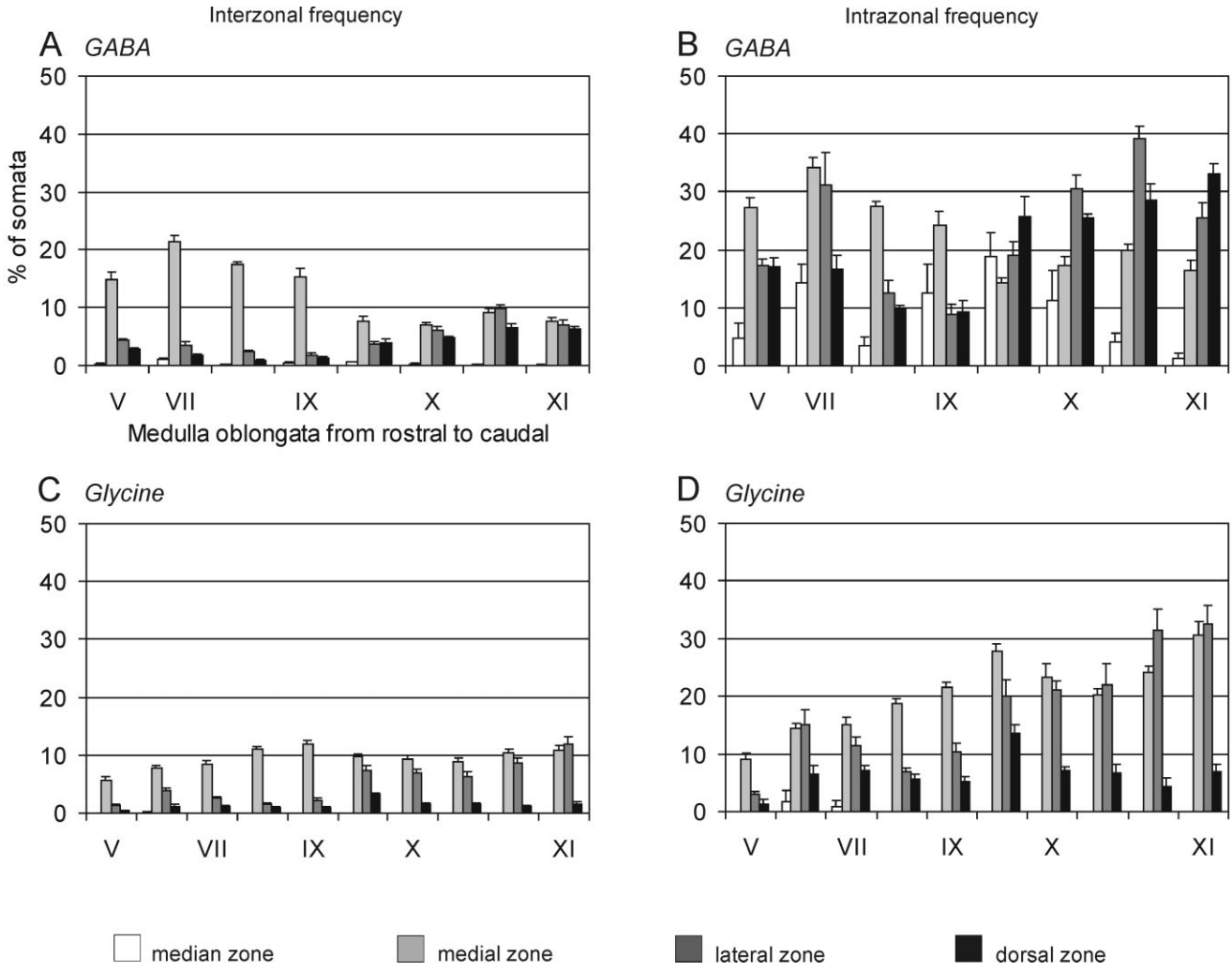


Fig. 3. Average interzonal (**A,C**) and average intrazonal frequencies (**B,D**) of GABA- and glycine-immunoreactive somata in one individual of *Plethodon shermani*. Each column represents 1) cell counts over a distance of 200 μm in one-half of the medulla, 2) in the case of interzonal frequencies the number of labeled somata in one longitudinal zone as percent of total cell number, and 3) in the case of intrazonal frequencies the number of labeled somata in one longitudinal zone as percent of the total cell number in the same zone. Roman numbers indicate the entrance of cranial nerves V–XI; vertical bars indicate standard error.

reactive somata, but is closely followed by the lateral and dorsal zone, while the amount of immunopositive somata in the median zone varies strongly. In the caudal medulla the intrazonal frequencies of GABA-ir somata are higher in the lateral and dorsal zone compared to the medial and median zone. The intrazonal frequencies of gly-ir somata are highest in the lateral and medial zone, followed by those in the dorsal zone, while the intrazonal frequency is lowest in the median zone.

In the following the average number and distribution of GABA- and gly-ir somata in each zone and throughout its rostrocaudal extent are presented. Examples of individuals are given in Figures 3–5. The majority of immunopositive somata were located in the rostral medulla oblongata; here, the largest number of GABA-ir and gly-ir somata was found in the medial zone. In the median zone, the number of GABA-ir neurons amounted to 13% (SE ± 1.46) on average of all somata in this zone, but increased to 16% (SE ± 1.47) at irregular intervals throughout the rostrocaudal extent (Figs. 3B, 4). Gly-ir somata were rarely found in the median zone (<1%) (Figs. 3D, 4). In the medial zone of the rostral medulla, 25% (SE ± 1.05) of all somata in this zone were immunolabeled for GABA and were mainly located in the periventricular part (Figs. 3B, 4, 5A). The number of GABA-ir somata in the medial zone decreased to 22% (SE ± 0.96) in the caudal medulla (Figs. 3B, 4, 6). In the rostral medial zone, 16% (SE ± 0.46) of gly-ir somata were labeled, and 18% (SE ± 0.54) were found in the medial zone of the caudal medulla; rostrally, somata formed a medial and a lateral cluster and were evenly distributed in the caudal medulla (Figs. 3D, 4, 5B,D). In the lateral zone of the rostral medulla oblongata, the number of GABA-ir somata amounted to 17% (SE ± 1.03), and for gly-ir somata the intrazonal frequency was 11% (SE ± 0.52) (Figs. 3B,D, 6). Somata with GABA-ir were evenly distributed and those with gly-ir formed one cluster (Figs. 4, 5A,B). In the caudal lateral

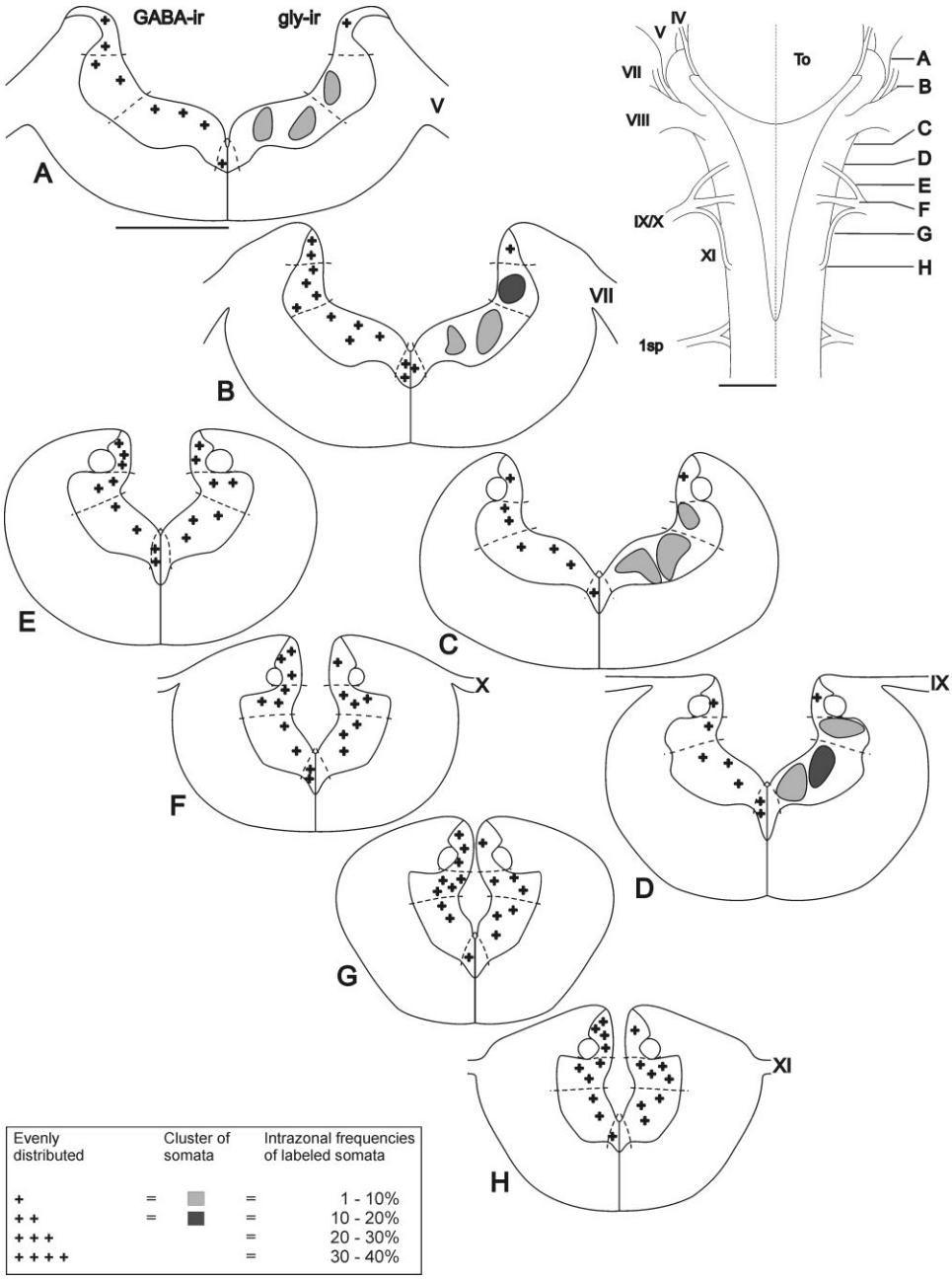
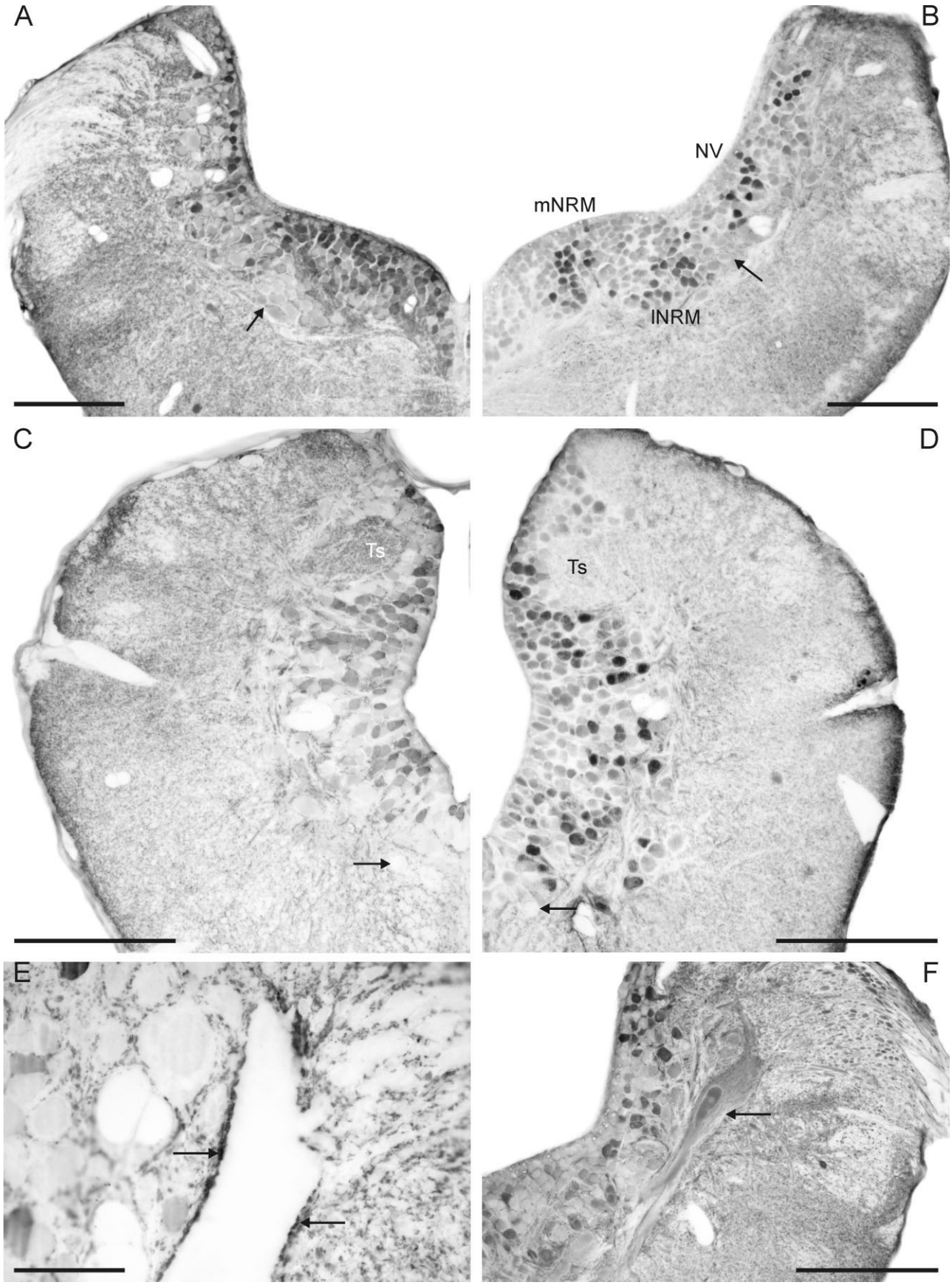


Fig. 4. Schematic drawings of transverse sections through the medulla oblongata (**A–H**) of *Plethodon shermani* (left and right side combined from two individuals) illustrating the intrazonal frequencies of GABA-ir (left) and gly-ir (right) somata in the gray matter. Section levels are indicated in the schematic dorsal view of the brainstem. Roman numbers designate cranial nerves; stippled lines indicate the border between the longitudinal zones, the open circle delimits the solitary tract (C–H). To tectum opticum. Scale bar = 500 μm.

zone, GABA-ir and gly-ir somata were evenly distributed, and their number increased continuously to 30% (SE ± 1.35) and 22% (SE ± 0.62), respectively (Figs. 3B,D, 4, 5C,D). The dorsal zone contained 19% (SE ± 1.06) of GABA-ir somata and 9% (SE ± 0.64) of gly-ir somata in the rostral medulla (Figs. 3B,D, 4, 5A,B). In the caudal medulla, the number of GABA-ir somata increased to 25% (SE ± 1.26) and remained constant for gly-ir somata (10%, SE ± 0.40) (Figs. 3B,D, 4, 5C,D).

Mauthner neuron

At the level of the entrance of the VIIIth cranial nerve, in *Plethodon* a Mauthner neuron was found bilaterally in the ventral gray matter (Fig. 5E,F), and its axon descended medially in the contralateral medulla oblongata (Fig. 5C,D). The size of the soma was 136 × 32 μm, while those of immunolabeled somata were 10 × 14 μm on average in *Plethodon*. The Mauthner neurons were weakly immunopositive for glycine (Fig. 5F) and immunonegative for GABA; but dense GABA-ir as well as moderate glycine-ir terminals covered the surface of the soma (Fig. 5E). In *Hydromantes*, a Mauthner neuron was not found. Diameters of immunopositive somata were 15 × 18 μm on average. In the colocalization experiments the descending axon of the Mauthner neuron was localized in the median zone of the medulla oblongata and was immu-


A
B
NV
mNRM
INRM
C
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Ts
E
F

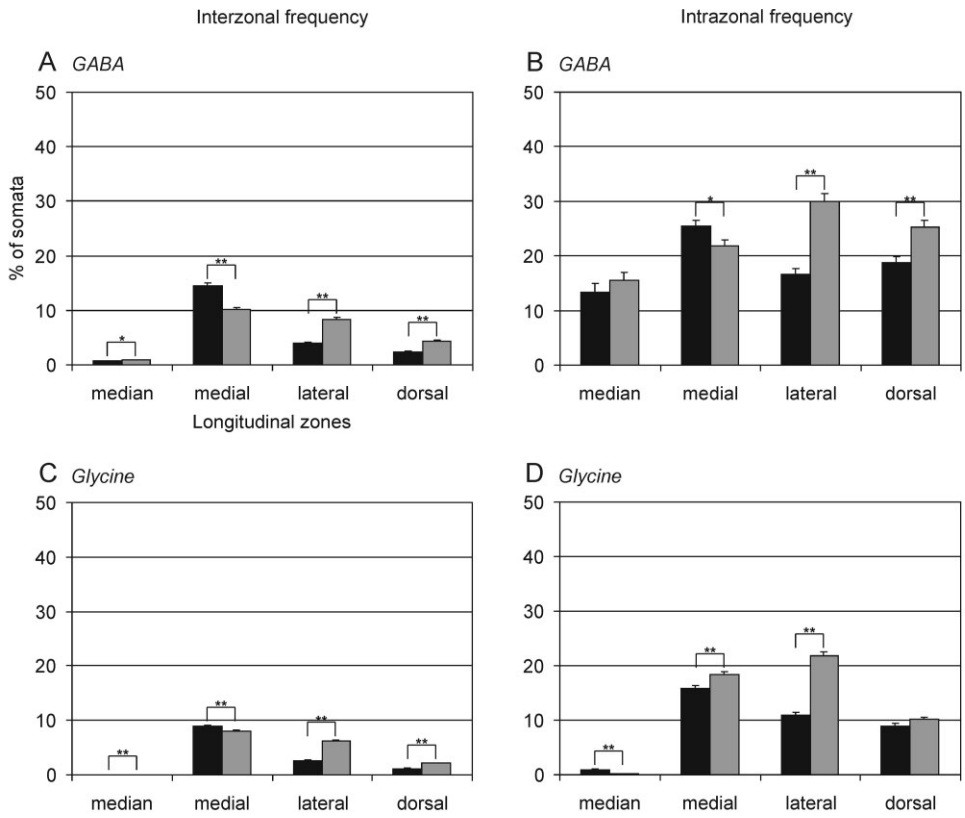

Fig. 6. Statistically significant differences between the rostral (black bars) and caudal (gray bars) average inter- or intrazonal frequencies of GABA- (**A,B**) and glycine-immunoreactive (**C,D**) somata in the four longitudinal zones in *Plethodon shermani* (n = 4 for each transmitter). Each column represents 1) cell counts over one half of the rostral or caudal medulla, 2) in the case of interzonal frequencies the average number of labeled somata in one longitudinal zone as percent of total cell number, and 3) in the case of intrazonal frequencies the average number of labeled somata in one longitudinal zone as percent of the total cell number in the same zone. Vertical bars indicate standard error. *$P < 0.05$; **$P < 0.01$.

noreactive for glutamate and weakly immunopositive for glycine. GABA-ir was not detected in the axon of the Mauthner neuron. The results of the immunohistochemical experiments performed on vibratome and semithin sections are in accordance.

Significance of differences in cell number

Since analysis using ANOVA showed no significant differences in the size of zones between individuals as well as between gender, data were collectively analyzed. Variances in inter- and intrazonal frequencies of GABA- and gly-ir somata across individuals are given by the standard error. *T*-test analysis revealed significant differences of the four longitudinal zones between the rostral and caudal medulla oblongata (Fig. 6). The average interzonal frequency of GABA- and gly-ir somata differed significantly in all longitudinal zones (Fig. 6A,C). The number of GABA-ir somata in the median zone of the caudal medulla was significantly larger ($P < 0.035$) compared to the rostral medulla, whereas that of gly-ir somata in the median zone and of GABA- and gly-ir somata in the medial zone was larger in the rostral compared to the caudal medulla, and differences were highly significant ($P < 0.01$). In the lateral and dorsal zones, differences between the rostral and caudal medulla were again highly significant ($P < 0.01$), but the interzonal frequency was larger for the number of GABA- and gly-ir somata in the caudal medulla. The distribution pattern of immunoreactive somata regarding the average intrazonal frequencies was the same except for gly-ir somata in the medial zone, which revealed a significant larger number in the caudal compared to the rostral medulla (Fig. 6B,D). Differences were not significant for GABA-ir somata in the median zone and for gly-ir somata in the dorsal zone, but were significant for GABA-ir somata in the medial zone ($P < 0.02$), and highly significant for GABA-ir somata in the lateral and dorsal zone as well as for gly-ir somata in the median, medial, and lateral zone ($P < 0.01$).

Fig. 5. Photomicrographs of transverse sections illustrating GABA-ir (**A,C,E**) and gly-ir (**B,D,F**) somata in *Plethodon shermani*. The distribution of immunolabeled somata is shown in the rostral (A,B) and caudal medulla (C,D). Arrows in A and B point to immunonegative clusters, those in C and D to the axon of the Mauthner neuron. E: High magnification of the soma of the Mauthner neuron covered with GABA-ir terminals (arrows). F: The soma of the Mauthner neuron (arrow) at the border between the white and gray matter in the lateral medulla oblongata reveals moderate glycine-immunoreactivity. mNRM, medial part; lNRM, lateral part of the nucleus reticularis medius; NV, vestibular nucleus; Ts, solitary tract. Scale bars = 200 μm in A–D,F; 50 μm in E.

Distribution of glutamate-like immunoreactivity

The interzonal and intrazonal frequency of glu-ir somata was determined only on semithin sections. The number of glu-ir neurons was higher than those of the two other transmitters. On average, the interzonal frequency was 2% (SE ± 0.21) in the median, 37% (SE ± 1.61) in the rostral medial, and 24% (SE ± 2.16) in the caudal medial zone. In the lateral zone, the interzonal frequency amounted to 13% (SE ± 0.61) in the rostral

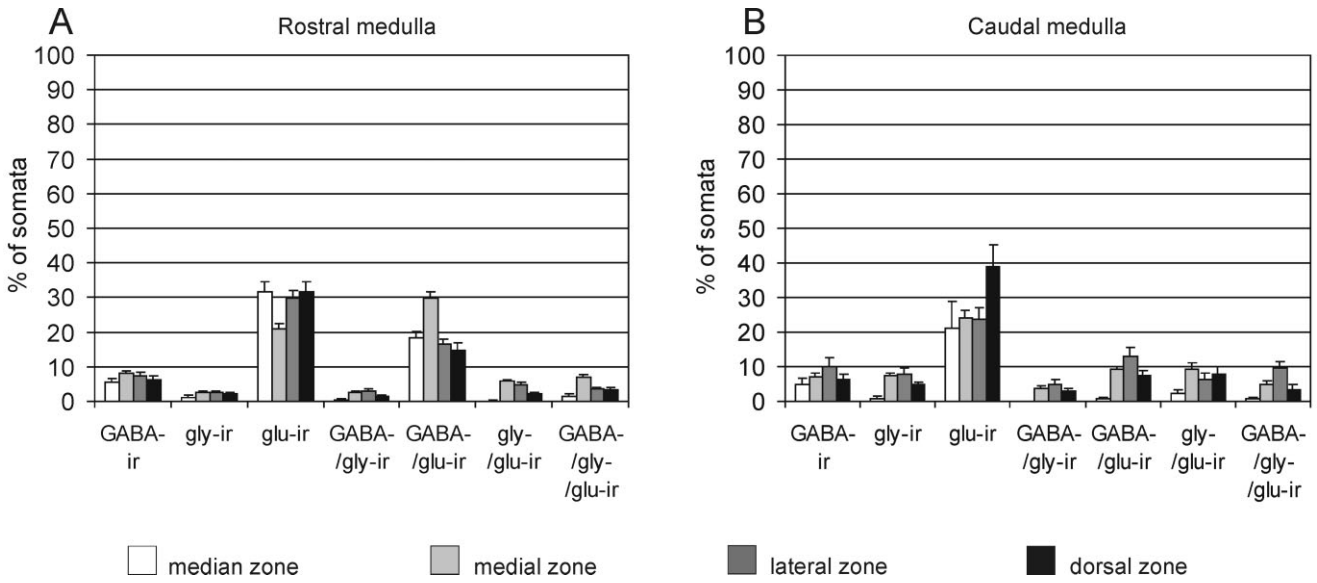

Fig. 7. Distribution of GABA-ir, gly-ir, and glu-ir somata in the rostral (**A**) and caudal (**B**) medulla oblongata in *Plethodon shermani* (n = 6). Average intrazonal frequencies of single transmitters and their dual or triple localization with other transmitters are shown. Each column represents the number of labeled somata in one zone as percent of total cell number in the same zone. Vertical bars indicate standard error.

and to 17% (SE ± 2.14) in the caudal medulla, and was 7% (SE ± 0.69) in the rostral dorsal and 12% (SE ± 2.09) in the caudal dorsal zone. In the rostral medulla, the average intrazonal frequency was 51% (SE ± 2.80) in the median, 64% (SE ± 2.75) in the medial, 55% (SE ± 2.65) in the lateral, and 52% (SE ± 4.09) in the dorsal zone. In the caudal medulla the intrazonal frequencies were 24% (SE ± 9.77) in the median, 47% (SE ± 4.28) in the medial, 53% (SE ± 7.02) in the lateral, and 58% (SE ± 9.75) in the dorsal zone.

Colocalization of GABA, glycine, and glutamate

The colocalization of the three transmitters was determined rostrally (level of Vth–IXth nerve) and caudally (level of Xth–XIth nerve) on semithin sections. The distribution of GABA and glycine was the same as described above using vibratome sections. In the following the average intrazonal frequencies of colocalizations are described (Fig. 7). In the rostral medulla oblongata (Fig. 7A), ~30% in the median, lateral, and dorsal, and 21% (SE ± 1.15) in the medial zone were exclusively glu-ir; 5–10% were exclusively GABA-ir and 1–2% exclusively gly-ir in each of the zones. Neurons with colocalization of GABA- and glu-ir were 30% (SE ± 2.0) in the medial zone and 15–20% in the other three longitudinal zones. Colocalization of GABA- and gly-ir as well as of gly- and glu-ir amounted to 5% in each longitudinal zone. Neurons with triple localization of transmitters ranged from 1–7% in the four longitudinal zones. In the caudal medulla (Fig. 7B), somata containing exclusively glu-ir again formed the largest group, with 39% (SE ± 6.16) in the dorsal zone and 23% in the median, medial, and lateral zone. The intrazonal frequencies of cell groups containing GABA-, gly-ir, dual or triple localization ranged from 2–13% in the different zones except for somata of the median zone, where they ranged from 0–5%. Somata containing only one transmitter and those containing two or three transmitters were found at all medullary levels investigated; an example is given in Figure 8. The pattern of colocalization of the three transmitters differs clearly along the dorsoventral axis in the rostral and caudal medulla. Neurons with GABA-ir are generally found in a periventricular position in all longitudinal zones, while most glu-ir neurons are localized in the intermediate portion of the cellular layer. Consequently, colocalization of GABA- and glu-ir somata is found predominantly there. Gly-ir neurons are mainly distributed in the ventral portion of the gray matter, especially in the medial zone, where they constitute two clusters, and a colocalization of glu- and gly-ir in neurons is found predominantly there.

Immunonegative somata

The intrazonal frequency of immunonegative somata in the rostral medulla was 46% (SE ± 3.34) in the median, 23% (SE ± 2.20) in the medial, 37% (SE ± 3.62) in the lateral, and 44% (SE ± 4.11) in the dorsal zone. In the caudal medulla, 70% (SE ± 10.42) of immunonegative somata were found in the median zone, followed by 48% (SE ± 9.31) in the dorsal and 35% in the medial and lateral zone. Cells lining the ventricle and constituting the ependymal layer were always immunonegative (Figs. 8, 9). Immunonegative somata were predominantly found in the ventral gray matter of the medial and lateral zone and were aligned close to the ventricle in the median and dorsal zone. Somata in the median zone and in the ventral medial and ventral lateral zone were located in clusters (Fig. 8); the latter two groups of immunonegative neurons had large somata with an average diameter of 18 × 24 μm in *Plethodon*.

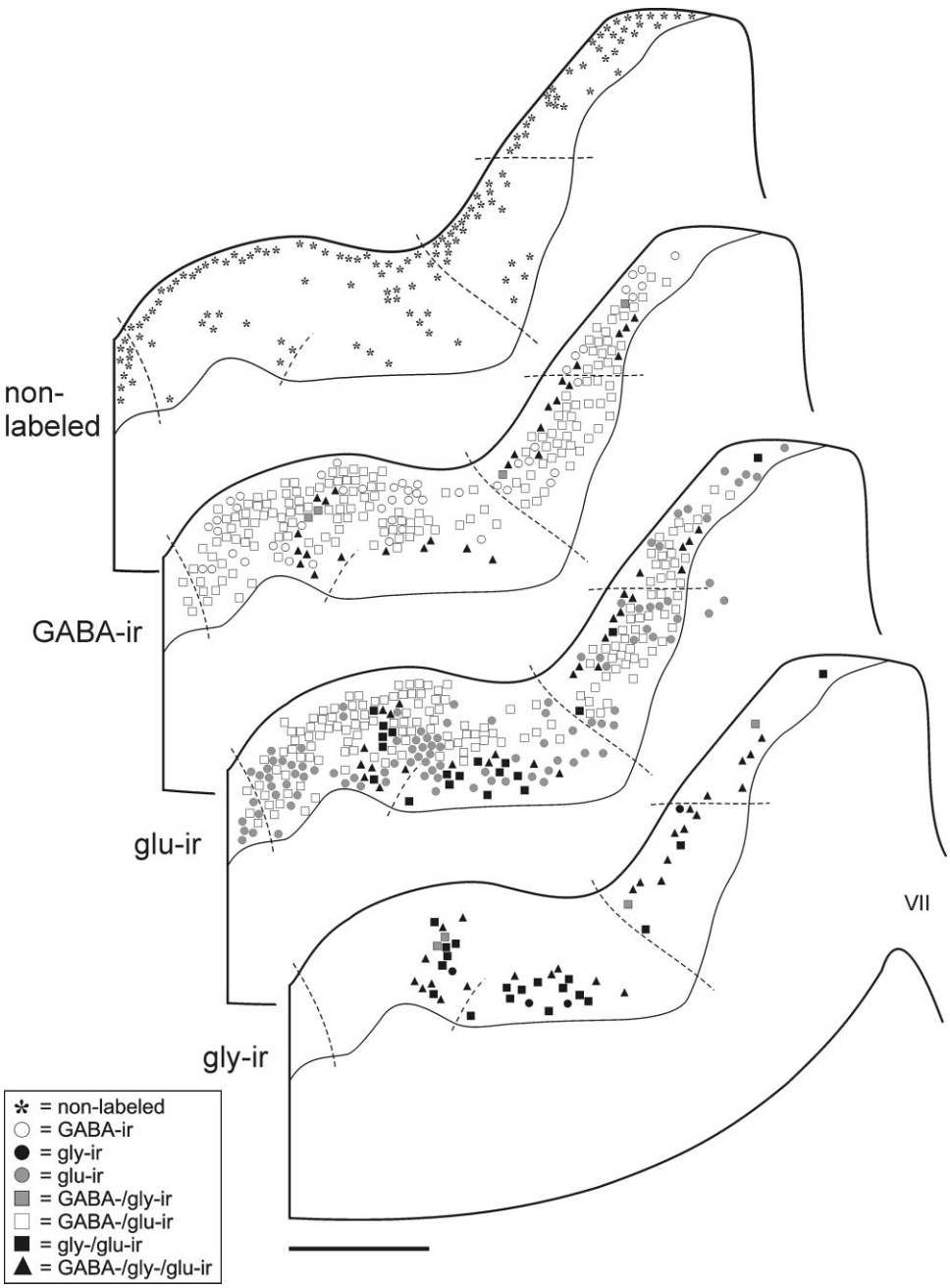

Fig. 8. Scheme of consecutive transverse sections showing the distribution of GABA-ir, gly-ir, and glu-ir somata and their colocalizations as well as of nonlabeled somata in the gray matter of the rostral medulla oblongata (*Plethodon shermani*). Stippled lines indicate the border between the longitudinal zones. VII, entrance of the VIIth cranial nerve. Scale bar = 200 μm.

Transmitter specificity of neurons with ascending projections to the tectum

After application of biocytin to the tectum mesencephali, neurons in the medulla oblongata were retrogradely stained. The majority of labeled neurons was found in the medial zone of the rostral medulla oblongata, mostly ipsilateral to the site of application, and formed a lateral and a medial cluster together constituting the NRM. The median zone contained only few labeled neurons situated only ipsilaterally, while in the lateral and dorsal zone labeled neurons were found ipsi- and contralaterally. Here neurons were dispersed within the gray matter. In the combined tracing-immunohistochemical experiment, 84% of labeled projection neurons of the NRM, located in the rostral medial zone of the medulla, were immunoreactive for glycine. An example of biocytin-labeled somata and their transmitter content is given in Figure 10. Approximately 17% of these neurons were exclusively glycinergic, 23% were colocalized with glutamate, and 38% showed a triple localization of the transmitters. Only a few (6%) projection neurons were immunoreactive for GABA and glycine. Differences are found in the transmitter distribution between the two parts of the NRM. Neurons of the lateral portion of the

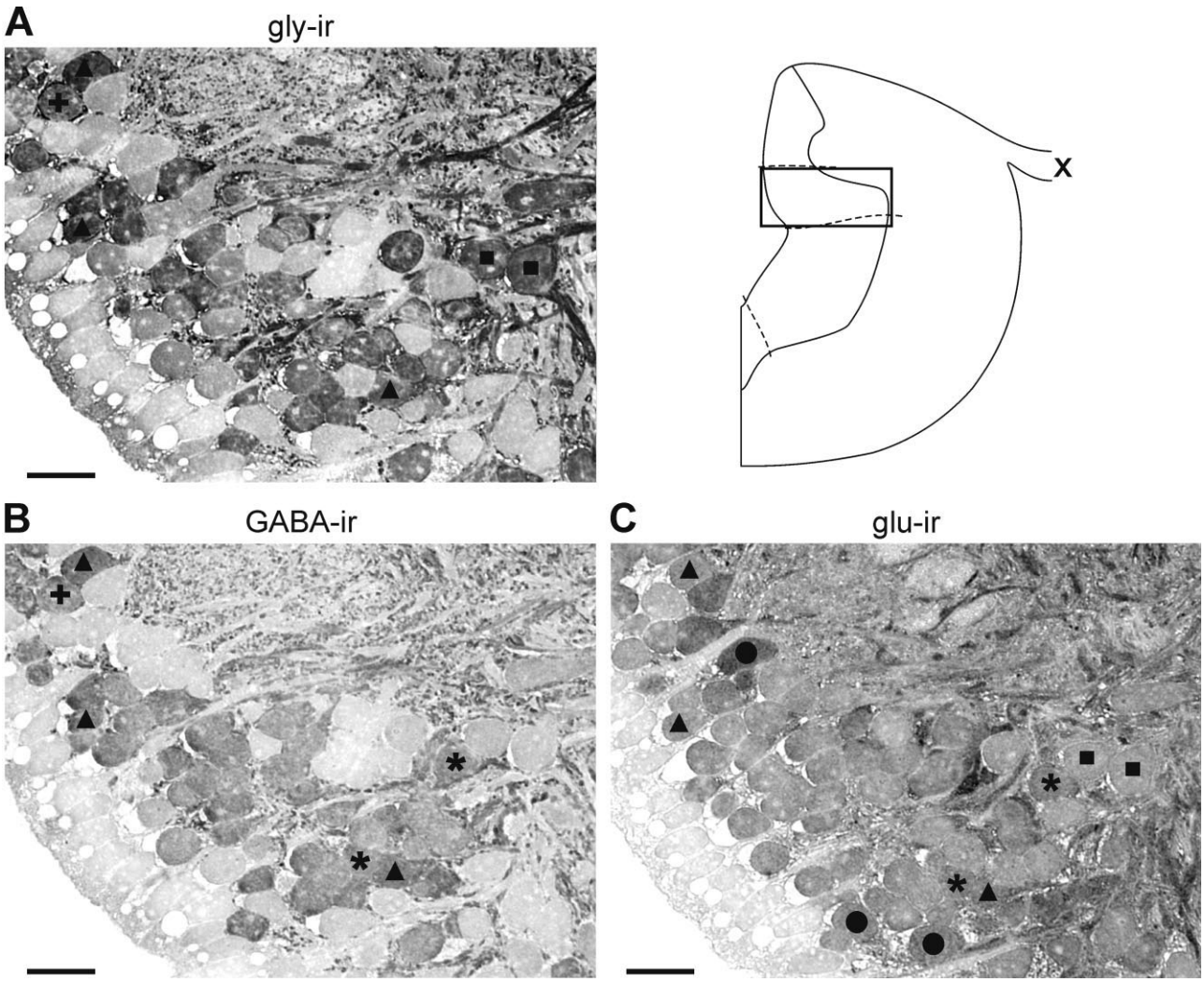

Fig. 9. Photomicrographs of consecutive semithin sections illustrating the distribution and colocalization of gly-ir (**A**), GABA-ir (**B**), and glu-ir (**C**) somata in the lateral zone of the caudal medulla oblongata (*Plethodon shermani*). The site of the presented region is demarcated by a rectangle in the schematic view of the medulla; stippled lines indicate the border between the longitudinal zones. Black dots characterize somata that are stained only for glutamate; crosses represent the colocalization of GABA- and gly-ir, and rectangles that of glu- and gly-ir. Asterisks denote somata with glu- and GABA-ir, arrowheads indicate localization of all three transmitters. Scale bars = 20 μm.

NRM were immunoreactive for glycine with a frequency of 91%; 19% of these neurons were exclusively glycinergic, 25% were colocalized with glutamate, 7% with GABA, and 40% revealed a triple localization of transmitters. In the medial portion of the NRM, 66% of the projection neurons were gly-ir and 13% exclusively glycinergic. With a frequency of 82%, glutamate is the main transmitter of the medial portion of the NRM. 6% of these latter neurons were exclusively glutamatergic, 24% were colocalized with GABA, and 19% with glycine; triple-localization of transmitters was found in 32% of neurons. In the lateral zone of the rostral medulla, 96% of the labeled projection neurons of the vestibular nucleus (NV) were glu-ir. Most of them (63%) were exclusively glutamatergic, 18% were colocalized with GABA, and 5% with glycine; 10% of NV neurons contained a triple localization of transmitters. In the rostral dorsal zone, labeled projection neurons of the dorsal nucleus (ND) were exclusively glu-ir.

DISCUSSION

The distribution of GABA-, gly-, and glu-ir neurons of the medulla oblongata was studied in plethodontid salamanders. We attempted to investigate the single dual and triple localization of transmitters in the longitudinal zones of the medulla, and to determine the transmitter specificity of neurons in the rostral medulla with reciprocal connections to the tectum. The distribution of transmitters in the medullary zones was the same in the two species and across individuals. Throughout the rostrocaudal extent of the medulla the average interzonal frequency of GABA-ir somata amounted up to nearly one-fourth of the total cell number, while that of gly-ir somata increased from roughly 10% in the rostral to 20% in the caudal medulla. In the rostral medulla the number of immunopositive neurons was highest in the medial zone and lower in the median, lateral, and dorsal zone. In the caudal me-

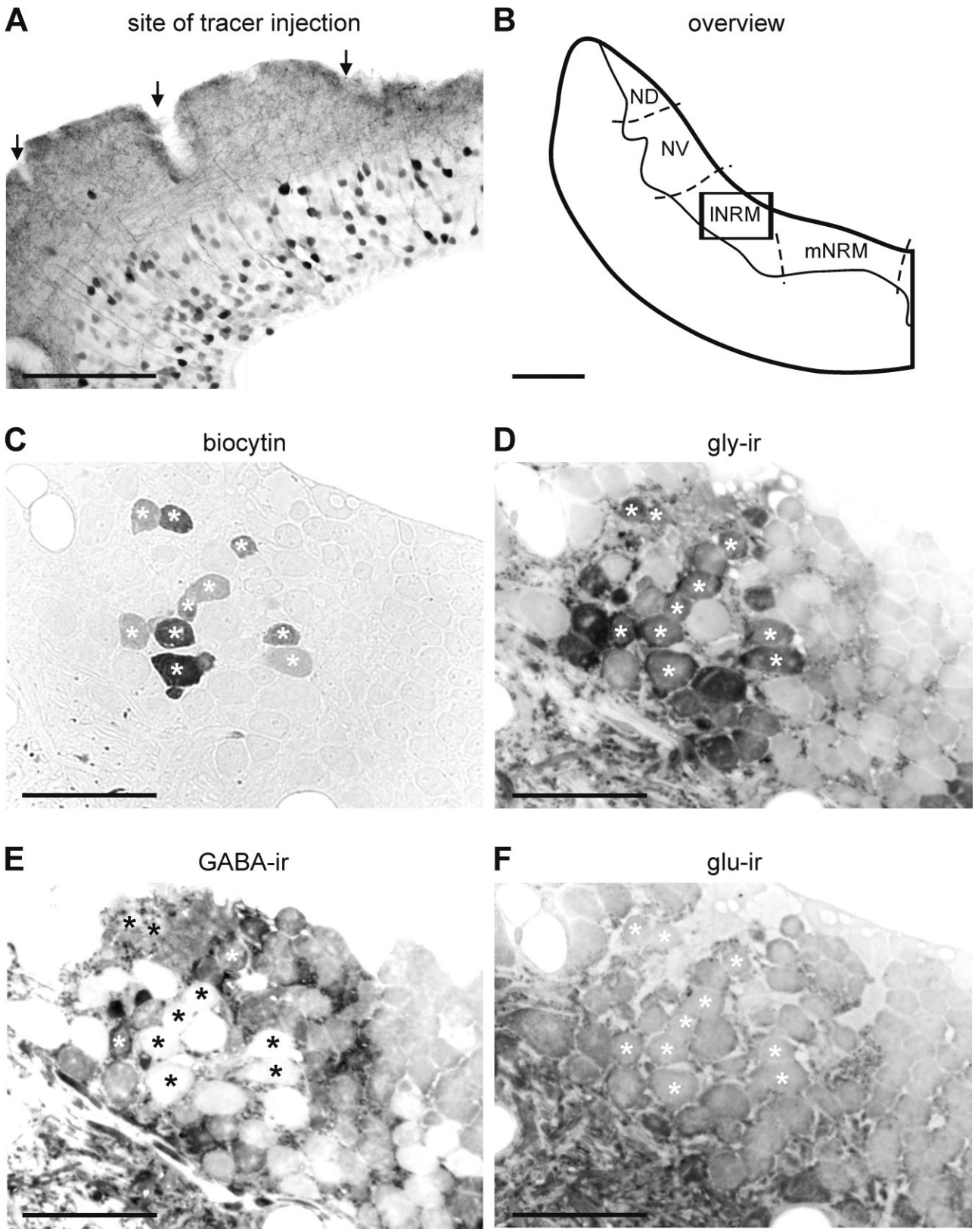

Fig. 10. Photomicrographs of transverse sections through the tectum and rostral medulla of *Plethodon shermani* showing retrogradely labeled neurons ipsilateral to the site of biocytin application and their transmitter content. **A:** Vibratome section illustrating the application site of biocytin in the tectum. Arrows point to the lesion sites. **B:** The location of the lateral part of the nucleus reticularis medius (lNRM) shown in C–F is indicated in the diagram of the medulla oblongata. Consecutive semithin sections stained for biocytin (**C**), glycine (**D**), GABA (**E**), and glutamate (**F**). White asterisks indicate biocytin-labeled and immunopositive somata, black asterisks designate biocytin-labeled immunonegative somata. mNRM, medial part of the nucleus reticularis medius; NV, vestibular nucleus; ND, dorsal nucleus. Medial is to the right, dorsal to the top. Scale bars = 200 μm in A,B; 50 μm in C–F.

dulla the medial, lateral, and dorsal zone contained roughly equal numbers of immunoreactive neurons. When intrazonal frequencies are considered, in the rostral medulla the largest number of GABA-ir and of gly-ir somata was again found in the medial zone, followed by immunoreactive cells in the lateral and dorsal zone. Gly-ir neurons were arranged in clusters in the medial and lateral zone and most of them were situated in the nucleus reticularis medius. Combined tracing and immunohistochemical experiments revealed that retrogradely labeled neurons of the NRM contained mainly the fast inhibitory transmitter glycine, often colocalized with glutamate. Retrogradely labeled neurons of the sensory vestibular and dorsal nucleus contained predominantly the fast excitatory transmitter glutamate. In the caudal medulla, the lateral and dorsal zone contained the largest amount of GABA-ir cells; gly-ir neurons were mainly found in the medial and lateral zone. Single, dual, and triple localization of GABA-, gly-, and glu-ir somata was present in all zones and throughout the rostrocaudal extent. Exclusively glu-ir neurons were most frequent, followed by neurons with a colocalization of GABA and glutamate. A Mauthner neuron revealing glu-ir and weak gly-ir was found in *Plethodon* but absent in *Hydromantes*.

Methodical considerations

The primary antibodies against GABA, glycine, and glutamate revealed specific binding as demonstrated by adsorption controls and controls of crossreactivity. While GABA is synthesized in neurons mainly as a transmitter, the amino acids glycine and glutamate are involved in numerous metabolic pathways in addition. However, an increased amount of glycine in neurons appears only as a transmitter, while glutamate is ambiguous with regard to its function (i.e., metabolic vs. transmitter). Furthermore, the colocalization of transmitters found in the present study differs in its significance. The colocalization of glycine and GABA as well as of glycine and glutamate can be interpreted as "true" colocalization, because the synthetic pathways of the respective transmitters are different. However, because glutamate is a precursor for GABA, the "true" quantity of colocalization of GABA and glutamate as two transmitters cannot be determined with certainty, and this is likewise the case for the ratio between the colocalization of GABA-glycine and of glutamate-glycine and for the colocalization of the three transmitters. In order to solve this problem, detection of glutamatedecarboxylase (GAD), which transforms glutamate into GABA, is necessary. However, these experiments could not be carried out because the commercially available antibodies against GAD were ineffective in the species studied. Nevertheless, we found that the number of colocalizations of glutamate and GABA did not exceed 30% on average, and this is the maximum number of neurons in which glutamate is possibly a precursor of GABA.

Longitudinal zones of the medulla oblongata

In amphibians the division of the medulla oblongata into four longitudinal zones is based mainly on the density and arrangement of cell masses and size and shape of somata, and this also holds for the division of the reticular formation into a median, medial, and lateral zone. (Herrick, 1948; Opdam and Nieuwenhuys, 1976; Nieuwenhuys and Opdam, 1976; Opdam et al., 1976; Nikundiwe and Nieuwenhuys, 1983). Nuclei of the reticular formation were recently investigated in *Rana pipiens* by combined tracing of bulbospinal neurons and immunohistochemistry of neuropeptides and serotonin, and were assumed to be homologous to the classical distinction of reticular nuclei established in mammals (Adli et al., 1999; Stuesse et al., 2001). In plethodontid salamanders, somata—except for giant cells such as Mauthner neurons—are more or less of the same size and are rather evenly distributed in the cellular layer; therefore, a classification based on the morphology and arrangement of cell masses is inadequate. However, using tracer and immunohistochemical investigations of viscero- and somatomotor nuclei, sensory afferents, and transmitter-specific nuclei, the longitudinal zones in the medulla oblongata of plethodontids match those of other amphibian species (Roth and Wake, 1985; Roth et al., 1988; Fritzsch, 1988, 1989; Dicke et al., 1997; Dicke and Mühlenbrock-Lenter, 1998).

Distribution of transmitters in the longitudinal zones

The distribution of immunopositive neurons was determined in the longitudinal zones and their interzonal and intrazonal frequencies were calculated. The interzonal frequency compares the number of immunolabeled cells across the different longitudinal zones, whereas the intrazonal frequency specifies the density of immunolabeled cells inside one zone. Thus, the interzonal frequency of immunostained neurons largely reflects the size of the different zones; consequently, the medial zone, being the largest one, contains the highest number of immunolabeled cells in the medulla, followed by the lateral, dorsal, and median zone. The intrazonal frequency better indicates the density of immunolabeled neurons because it is independent of the size of the different volumes.

Median zone

Within the median zone, glu-ir neurons were most frequent; fewer somata exhibited GABA-ir, and gly-ir somata were rarely found in the rostral medulla. Because GABA-ir and gly-ir neurons were mainly dispersed, the majority of them probably are interneurons. GABA-ir somata were also detected in the rostral median zone of *Rana esculenta* and *Triturus cristatus carnifex* (Franzoni and Morino, 1989), whereas in *Pleurodeles waltl* and *Triturus alpestris* none were found in this zone (Naujoks-Manteuffel et al., 1994). The median zone includes the serotonergic raphe nuclei (Clairambault et al., 1994; Dicke et al., 1997; Adli et al., 1999; Stuesse et al., 2001); accordingly, in the present study immunonegative cells were arranged in clusters. In mammals, GABA-ir, glu-ir, or GAD-ir somata were labeled in the nucleus raphe magnus located in the rostral median zone, and only a few of these somata were found in the nuclei raphe obscurus and pallidus of the caudal medulla (Reichling and Basbaum, 1990; Jones et al., 1991; Jones, 1995; Fredette et al., 1992; Holmes et al., 1994; Li et al., 1996, 1997; Kalyuzhny and Wessendorf, 1997; Ellenberger, 1999; Maloney et al., 2000). Somata containing gly-ir were localized only in the nucleus raphe magnus of rats (Li et al., 1996, 1997; Rampon et al., 1996, 1999). Thus, the occurrence of transmitters and their distribution in neurons of the mammalian median zone are in accordance with the results of the present study in plethodontid salamanders. It remains undecided whether the glu-ir somata are colocalized with

5-HT as described in other vertebrates (overview in Hökfelt et al., 2000).

Medial zone

Within the rostral medulla the medial zone reveals the highest density of GABA-, gly-, and glu-ir somata, and consequently a greater amount of colocalization of the three transmitters. A high density of GABA-ir somata was also found in the medial zone of the medulla oblongata of other amphibian species (Franzoni and Morino, 1989; Naujoks-Manteuffel et al., 1994). GABA- and gly-ir somata occupy different positions along the dorsoventral axis and gly-ir somata were located in two clusters. Many of these neurons projected to the tectum mesencephali and belong to the NRM, which has been described in a tracer study in *Plethodon* and *Hydromantes* (Dicke and Mühlenbrock-Lenter, 1998). Because of the similar location of the NRM and its projection pattern to the tectum, this nucleus most likely is homologous to the medial rhombencephalic reticular nucleus of goldfish (Pérez-Pérez et al., 2003a,b). In amphibians as well as in other vertebrates the transmitter specificity of ascending projections of reticular nuclei in the medial zone has not been studied so far. Intracellular staining of neurons of the NRM in plethodontids revealed descending projections to the spinal cord in addition to ascending projections (Heimbuch and Dicke, 1998; Heimbuch, 2001), and tracing experiments in different species of the amphibian orders likewise reported descending projections of the middle reticular nucleus (Sánchez-Camacho et al., 2001a). In studies on the distribution of neuropeptides and projections of the reticular formation in *Rana pipiens* (Adli et al., 1999; Stuesse et al., 2001), a magnocellular, a gigantocellular, and a paragigantocellular reticular nucleus with descending projections to the medulla spinalis and located in the medial zone of the rostral medulla was homologized with that of rats. Although the distribution pattern of amino acid transmitters and of neuropeptides in the medulla oblongata differs, the NRM of plethodontids might be homologous to one of these nuclei found in frogs and rats. Vertes et al. (1986) described ascending projections from the gigantocellular reticular nucleus as well as from the alpha part of the gigantocellular reticular nucleus to the deep and intermediate fiber layers of the superior colliculus, the brain region homologous to the tectum. Since these ascending projections resemble the ascending projection pattern of the NRM of the present study, the NRM likewise appears to be homologous to the gigantocellular reticular nucleus and/or to the alpha part of this nucleus. In mammals, glycinergic neurons have been found in the region of both gigantocellular nuclei (Li et al., 1996, 1997; Rampon et al., 1996, 1999), and GABA- as well as GAD-ir somata were also localized in these two regions (Reichling and Basbaum, 1990; Li et al., 1996, 1997; Ellenberger, 1999; Holmes et al., 1994; Jones et al., 1991; Jones, 1995; Maloney et al., 2000). The immunonegative cell clusters found in the transition zone between the gray and white matter correspond with the position of cranial motor neurons, which have been characterized by retrograde labeling in *Plethodon* and *Hydromantes* as well as by immunohistochemistry of cholineacetyltransferase in other amphibian species (Roth et al., 1988; Wake et al., 1988; Marín et al., 1997).

Lateral zone

In *Plethodon* and *Hydromantes* the lateral zone is characterized by a low density of the three transmitters in the rostral part and a high density in the caudal part. The NV situated in the lateral zone also contained neurons projecting to the tectum, which is in accordance with the tracer study of Dicke and Mühlenbrock-Lenter (1998). The NV exhibited a number of glu-ir projection neurons, which were often colocalized with glycine and/or GABA. Reichenberger et al. (1997) investigated the colocalization of GABA-, gly-, and glu-ir in the vestibular nuclear complex of *Rana temporaria* and found that within the vestibular nuclear complex most neurons were immunoreactive for glutamate and colocalizations with GABA and/or glycine were demonstrated. This is consistent with results of the present study. Reichenberger et al. (1997) assume some of these neurons to be projection neurons, which is demonstrated for the vestibular neurons of *Plethodon* in the present study. Also, the immunochemical results of the NV in plethodontids largely correspond with the distribution of amino acid transmitters in the vestibular nuclei of mammals. Glycinergic neurons as well as GAD-immunoreactive somata have been reported in studies of the vestibular nuclei in different mammalian species (Jones et al., 1991; Li et al., 1996, 1997; Rampon et al., 1996; Ellenberger, 1999). Walberg et al. (1990) demonstrated a vast majority of glutamatergic neurons in the vestibular nuclei of the cat, and found that colocalizations of two or more neuroactive amino acids such as GABA, glycine, glutamate, and aspartate is rather the rule than an exception in vestibular neurons of the cat. In the caudal medulla, part of fibers of the solitary tract located at the border of the lateral and dorsal zone as well as neurons forming the solitary nucleus and surrounding the tract rostrocaudally have been identified as catecholaminergic in a number of frog and salamander species (Gonzaléz and Smeets, 1991, 1993, 1994; González et al., 1994, 1995; Sánchez-Camacho et al., 2001b). The relatively homogeneous distribution of GABA, glycine, and glutamate of this region found in the present study indicates that the majority of immunopositive neurons are interneurons that surround catecholaminergic projection neurons.

Dorsal zone

In the dorsal zone of the rostral medulla oblongata the projection neurons of the ND described by Dicke and Mühlenbrock-Lenter (1998) revealed only glutamate immunoreactivity in the present study. The ND is involved in the processing of acoustic stimuli, and its main sensory input are low frequency and substrate-borne vibrations mediated by afferents of the VIIIth nerve (Roth et al., 1993). In salamanders, anterograde labeling of the dorsal and vestibular nuclei revealed an ascending projection to the torus semicircularis, the main center for auditory and vestibular processing, and to the tectum (Will, 1988; Dicke and Mühlenbrock-Lenter, 1998). It remains to be investigated whether neurons of the ND and NV project to the torus and the tectum in parallel, or whether the projections to these targets originate from separate populations. Therefore, in the present study the glu-ir of the projection neurons in ND and NV can only be attributed to those projecting to the tectum. The glu-ir projection neurons of the ND are surrounded by GABA- and gly-ir somata, which most likely appear to be interneurons; however, it

cannot be excluded that some of them belong to nonlabeled neurons projecting to the torus semicircularis. In ranid frogs and in *Pleurodeles waltl,* projection neurons with descending projections were rarely found in the dorsal zone of the rostral medulla oblongata (Adli et al., 1999; Sánchez-Camacho et al., 2001a; Stuesse et al., 2001). In *Rana esculenta, Pleurodeles waltl*, and *Triturus alpestris*, the dorsalmost portion of the rostral medulla was largely void of GABA-ir somata (Franzoni and Morino, 1989; Naujoks-Manteuffel et al., 1994), whereas some GABA-ir somata were labeled in the acoustico-lateral area; this latter region is comparable to the transition area between the lateral and dorsal zone of the present study. The small amount of GABA-ir somata in the two studies mentioned is inconsistent with an intrazonal frequency of roughly 20% of GABA-ir neurons in the rostral dorsal medulla of the present study.

Functional considerations

Ascending projections in plethodontids were demonstrated by a tracer study of Dicke and Mühlenbrock-Lenter (1998). In the rostral medulla, neurons of the NV and ND project to the contralateral tectal fiber layers 4 and 5, while the NRM gives rise to an extensive ipsilateral ascending fiber projection, which not only extends in fiber layer 4, but also reaches the deep fiber layers 5 and 7, and collaterals extend into fiber layer 3 (Dicke and Mühlenbrock-Lenter, 1998; Dicke, unpubl. data). According to our results, almost all projection neurons of the NRM are glycinergic, but at the same time exhibit a complex pattern of glutamate and GABA colocalization. The dominant glycinergic component is further substantiated by the fact that in *Plethodon* a gly-ir fiber bundle ascends from the ventral medulla and extends in the tectal fiber layers 4 and 5, and few tectal cells in the upper row of the cellular layers 6 and 8 show immunoreactivity for glycine (Wallstein and Dicke, 1996). The function of this glycinergic reticulo-tectal projection is assumed to be modulatory: in vitro pharmacological studies demonstrated that during recording from amphibian tectal cells at electrical stimulation of the optic nerve, application of glycine produced a biphasic effect. Low concentrations of glycine had an excitatory and high concentrations an inhibitory effect (Sivilotti and Nistri, 1986; Kahl, 1999). The medial zone of the medulla oblongata and the tectum appear to strongly influence each other because the tectum sends strong axon bundles to the medial zone, and these tectobulbar tracts form numerous collaterals towards medullary somata (Dicke, 1999). The reticulo-tectal interactions appear to be organized in a complex manner, because serotonergic axonal projections from the median rostral medulla (i.e., the raphe nuclei) to the NRM of the medial zone as well as to the fiber layer of the tectum exist (Dicke et al., 1997). Modulatory effects of the visual surround on tectal information processing have been found during object selection in the orienting behavior of *Plethodon* (Schuelert and Dicke, 2002) and in electrophysiological recordings from tectal neurons (Schuelert and Dicke, 2005). In this context, the NRM appears to be part of a feedback loop between tectum and medulla and is a candidate for the control of alertness and vigilance in visuomotor behavior. In goldfish, Pérez-Pérez et al. (2003a) likewise described strong reciprocal connections between the tectum and the medial rhombencephalic reticular nucleus, the probable homolog of the NRM. The authors assume that a negative feedback loop exists between these two brain structures. The result of our study gives further evidence that the feedback loop between medulla and tectum is mediated by glycine. In monkeys, reciprocal connections between the superior colliculus and the mesencephalic reticular formation have been reported that might constitute a local feedback circuit for eye movements (Chen and May, 2000). Another function of glycine in medial reticular neurons might be the co-release of glycine and glutamate at NMDA-synapses in the tectum, since most of the gly-ir somata of retrogradely labeled projection neurons were also immunoreactive for glutamate. This co-action might constitute the excitatory component of the glycinergic biphasic effect mentioned above. Besides this proposed inhibitory or modulatory influence of the NRM, the tectum is also innervated by excitatory projection neurons of the NV and ND to the deep tectal fiber layer, which most likely initiates sensory and multimodal processing in the tectum.

Differences in the species investigated

Another important functional aspect concerns the fundamental similarity of the data obtained in *Plethodon* and *Hydromantes*, despite the fact that these two species belong to different tribes of the family Plethodontidae and have different feeding mechanisms and strategies. From the latter fact we can conclude that the premotor and motor centers situated in the medulla oblongata generate different spatio-temporal patterns of muscular activation for feeding using the same basic networks. One exception is that in *Plethodon*, but not in *Hydromantes*, a Mauthner neuron was found, which was immunoreactive for glutamate and weak immunopositive for glycine, but was innervated by dense GABA-ir as well as moderate gly-ir terminals. An innervation by GABA-ir, GAD-ir, or gly-ir terminals on the surface of the Mauthner neuron has been reported in teleosts (Petrov et al., 1991; Lee et al., 1993; Triller et al., 1993; Sur et al., 1994). In the salamander *Pleurodeles waltl* the Mauthner neuron was immunoreactive for ChAT (Marín et al., 1997). This finding is not consistent with the situation found in *Plethodon* (unpubl. data, U. Dicke) and in teleosts, where ChAT has not been found in Mauthner neurons (Rhodes et al., 1986; Ekström, 1987). In anamniotes, Mauthner neurons are involved in the control of escape behavior, but also—as was demonstrated in the goldfish—of other motor actions in the context of social behavior, parental care, and feeding (Will, 1991; Canfield and Rose, 1993; Eaton et al., 2001). The presence of a Mauthner neuron in *Plethodon* is remarkable in light of the fact that this species, like *Hydromantes*, is fully terrestrial and completely lacks an aquatic larval phase. However, during its embryonic development *Plethodon* as well as the other members of the plethodontid tribe Plethodontini still forms aquatic larval structures, while in *Hydromantes* and the other members of the plethodontid tribe Bolitoglossini all traces of larval structures during embryonic development have disappeared, due to secondary simplification in the context of pedomorphosis (Roth et al., 1993). The function of the Mauthner neuron in *Plethodon* is unknown, partly because its connectivity within the medulla oblongata has not been studied.

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