Serotonin Neurons Project to Small Blood Vessels in the Brain

Abstract. *Electrolytic lesions of the nucleus raphe dorsalis and medianus reduce the concentration of serotonin (5-hydroxytryptamine) within rat brain intraparenchymal blood vessels. The concentration of serotonin within these vessels increases or decreases after the administration of drugs that modify the biosynthesis and degradation of serotonin or destroy nerve terminals by an uptake-dependent mechanism. These studies provide evidence for the existence of a serotonin-containing pathway seemingly analogous to the neuronal projection that terminates on small parenchymal blood vessels from noradrenergic neurons of the locus coeruleus.*

Cerebral blood vessels are richly innervated by noradrenergic neurons whose cell bodies are located within the autonomic nervous system [superior cervical ganglia (*1*)] and brainstem [locus coeruleus (*2*)]. Nerve plexuses that contain other neurotransmitters [for example, serotonin, substance P, neurotensin (*3*), vasoactive intestinal polypeptide (*4*)] have recently been visualized by immunocytochemical and autoradiographic techniques; however, little is known about the origin of these neurons or of their relationship to the physiology of the cerebral circulation. Among neurotransmitters, serotonin (5-hydroxytryptamine) appears to be the most potent vasoactive substance when applied in vitro to large vessels (*5*), in vivo to pial vessels (*6*), or when injected into the carotid artery (*7*). In some situations, serotonin causes constriction of cerebral blood vessels (*8*); in other cases, dilatation (*9*). Besides exhibiting effects directly on vascular smooth muscle (*10*), it has been postulated that serotonin modifies vascular reactivity via brainstem mechanisms (*11*). For these and other reasons, serotonin has frequently been implicated in the pathogenesis of a number of vascular disorders, including migraine and ischemia (*12*). The development of methods for isolating an enriched preparation of brain microvessels (*13*) has made possible the biochemical and pharmacological studies we now report. Our studies demonstrate that serotonin-containing neurons arising within the midbrain raphe nuclei project to small intraparenchymal blood vessels within the brain.

Sprague-Dawley rats (Charles River) were caged under diurnal lighting conditions (lights on from 8 a.m. to 8 p.m.) and had free access to rat and mouse formula (Charles River). Brain microvessels were prepared from rat forebrains according to a modification of previously published methods (*14*). The tissue, approximately 2 mg from each forebrain, consisted of arterioles, capillaries, and venules. Microvessel samples were found to be free of contaminating glia, nerve cell bodies, mast cells, synaptosomes, and platelets (*15*).

Serotonin was measured by radioenzymatic microassay (*16*) modified by extracting the reaction mixture into chloroform (instead of toluene) and by applying this extract to unidimensional multiple development thin-layer chromatography (*17*). We were thus able to measure as little as 100 fmole of this indoleamine in several tissues and body fluids (*18*). Microvessel samples prepared from 450-g rats contained 10 pmole of serotonin per milligram of protein, which agrees with values obtained previously from bovine cerebral cortical microvessels (*19*). This amount did not change when rats were perfused with 75 ml of iced saline by intracardiac injection or after rats were made severely thrombocytopenic (Table 1). These results suggest that serotonin is present within the walls of cerebral microvessels—a finding previously reported in larger blood vessels of the peripheral circulation (*20*).

Virtually all of the serotonin-containing neurons within the brain arise from cell bodies located within the brainstem nuclei of the raphe system. The nucleus raphe dorsalis and medianus are located within the midbrain and project widely to many forebrain structures (*21*). Large lesions within these nuclei cause major and moderate reductions in the concentration of serotonin within telencephalon and diencephalon, respectively (*22*). To determine whether microvessel serotonin is synthesized within cells of the midbrain raphe nuclei (*11*), ether-anesthetized male rats (300 g) were subjected to stereotaxically placed electrolytic lesions of the dorsal and medial raphe nuclei (*23*). A second group was anesthetized and craniotomized, and 2 weeks later, microvessels and a small amount of right frontal cortex were assayed for serotonin. Brainstems were examined by light microscopy to confirm the location of the lesion. As is indicated in Table 2, dorsal and medial lesions produced a 70 percent reduction in microvessel serotonin concentration as compared to controls that were given sham operations. A third group was subjected to bilateral superior cervical ganglionectomy. Compared with controls, the decrease in microvessel serotonin in this group is not statistically significant (Table 2). The failure of ganglionectomy to modify microvessel serotonin content, and the ability of raphe lesions to reduce these concentrations, provide evidence that serotonin is not stored within noradrenergic terminals but is probably contained within serotoninergic nerve endings.

The possibility remains that other raphe nuclei also project to brain microvessels (note in Table 2 that serotonin concentration in microvessels decreased by 70 percent, whereas cortical levels decreased by more than 90 percent). Some support for this possibility is provided by data showing that stimulation of the nucleus raphe obscurus in the lower brainstem significantly increases blood flow in the brain (*24*). An alternative possibility is that mast cells [noted to surround brain blood vessels (*25*)] were present in our sample despite our inability to identify them with special stains.

The enzyme tryptophan hydroxylase appears to be confined primarily to serotoninergic neurons (*26*), not to sites of serotonin storage such as blood platelets (*27*). In brain tissue, and perhaps in other tissues, tryptophan hydroxylase is not normally saturated with its substrate, and the estimated affinity constant (K_m) exceeds the concentrations of tryptophan found under physiological conditions (*28*). Hence, variations in substrate saturation levels alter the rate at which serotonin is synthesized in vivo (*29, 30*). Table 3 shows that 1 hour after rats were injected with L-tryptophan (125 mg/kg), or diluent, the level of serotonin increased above control values in microvessels and right frontal cortex, but not

Table 1. Serotonin concentrations in brain microvessels from rats perfused with saline or injected with antiserum to platelets. In treatment with antiserum to platelets, 500-g male Sprague-Dawley rats were injected twice daily with 0.25 ml of sheep antiserum to rat platelets until blood platelet concentrations decreased below 20,000 per cubic millimeter (five injections) (*36*). On day 3, the animals were decapitated and microvessel preparations were made from whole forebrains. In saline perfusion treatment, the rats were anesthetized with ether and perfused with at least 60 ml of iced saline via the left ventricle after right auriculectomy. Microvessel preparations were then made.

Treatment	Serotonin levels (pmole/mg protein)*
Control (N = 12)	10 ± 2
Saline perfusion (N = 12)	9 ± 2†
Antiplatelet serums (N =5)	8 ± 3†

*Data are expressed as means ± S.E.M. †Not significant ($P \geq .1$).

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in plasma. When animals were injected with *p*-chlorophenylalanine (300 mg/kg) (*31*), a competitive inhibitor of this enzyme, significant decreases in microvessel serotonin content were measured 24 hours after drug treatment (Table 3). These results are indirect evidence that tryptophan hydroxylase is present within brain blood vessels, where it is probably unsaturated with substrate. The extent to which physiological changes in the plasma and brain levels of tryptophan [for example, after eating (*30*)] are able to modify the synthesis and perhaps release of serotonin within vascular nerve endings deserves additional study.

To further clarify the relationship between serotoninergic nerve terminals and brain microvessels, rats were injected with *p*-chloroamphetamine (*p*-CA; 10 mg/kg, intraperitoneally), a neurotoxin whose pharmacological activity depends on specific uptake and reuptake into serotonin-containing nerve endings (*32*) or saline vehicle. After 24 hours, microvessel samples were analyzed again for their content of serotonin. Serotonin levels among *p*-CA-treated animals decreased by 88 and 40 percent in cortex and microvessels, respectively. These results provide additional support for the existence of serotonin-containing nerve terminals within the brain microvessels; they also suggest that such terminals have the capacity to take up neurotransmitter, an observation that agrees with preliminary findings obtained by light microscopic autoradiography (*33*). Other drug experiments on the effects of pargyline, a monoamine oxidase inhibitor, confirm previous reports that brain microvessels possess significant amounts of this enzyme (*34*) (Table 3).

In conclusion, we propose that small blood vessels in the brain contain serotoninergic neurons whose cell bodies are located within the nuclei of the raphe system. These neurons may function as chemoreceptors to detect changes in tissue and blood composition. They may also regulate blood flow and vascular permeability [functions that were also proposed for the central noradrenergic nervous system (*35*)], and thereby provide the brain with a mechanism for controlling its own microcirculation.

Table 2. Serotonin concentrations in brain microvessels from rats after electrolytic lesions of the raphe nuclei or superior cervical ganglionectomy. In lesions of dorsal and medial raphe nuclei, 350-g male Sprague-Dawley rats were anesthetized with ether and placed in a Kopf stereotaxic apparatus with the incisor bar positioned 3.5 mm above the interaural plane. Lesions were induced with a Nichrome wire electrode (0.43-mm diameter, insulated with Beldenamel) set at an angle of 10° from the vertical axis. To destroy the nucleus raphe dorsalis, a current of 2 mA was applied for 20 seconds to an electrode introduced 1.2 mm lateral to the midsagittal line, 1.5 mm anterior to the lambda, and 7.0 mm below the skull. To lesion the nucleus raphe medianus, a similar current was applied for 25 seconds to an electrode 1.5 mm lateral to the midsagittal suture, 1.5 mm anterior to the lambda, and 8.9 mm below the surface of the skull. Two weeks later, microvessels were prepared from whole forebrains and serotonin levels were determined in this tissue and in a small amount of right frontal cortex. Sham-operated animals were treated by ether anesthesia and craniotomy without electrode placement. In (bilateral) superior cervical ganglionectomy, rats were subjected to surgery under ether anesthesia and, at least 4 weeks later, were decapitated for microvessel preparation.

Treatment	Serotonin levels (pmole/mg protein)*	
	Microvessels	Cortex
Sham surgery (control group; $N = 12$)	10 ± 2	35 ± 3
Lesion of dorsal and medial raphe nuclei ($N = 11$)	3 ± 1†	3 ± 1†
Superior cervical ganglionectomy ($N = 5$)	11 ± 1	32 ± 4

*Data are expressed as means ± S.E.M. †$P \leq .05$.

Table 3. Serotonin levels in rat brain microvessels, cerebral cortex, and blood platelets after various pharmacological treatments. Male Sprague-Dawley rats (300 to 450 g) were injected with L-tryptophan, *p*-chlorophenylalanine (*p*-CPA), *p*-chloroamphetamine (*p*-CA), or pargyline. Control animals for the experiments involving L-tryptophan received 0.5 percent carboxymethylcellulose (2 ml/kg); all other control animals received saline vehicle in the same volume. L-Tryptophan- and pargyline-treated animals were decapitated 1 hour after drug treatment, whereas *p*-CA- and *p*-CPA-treated animals were killed after 24 hours. Microvessels and cerebral cortex were assayed for serotonin (*16–18*); levels in platelets were determined by fluorometric assay (*37*) after platelet pellets were prepared from whole blood treated with NIH Formula A. Data are expressed as serotonin per milliliter of platelet-rich plasma.

Treatment	Dose (mg/kg)	Serotonin level*		
		Microvessels (pmole/mg protein)	Cortex (pmole/mg protein)	Platelets (ng/ml)
Control ($N = 14$)		10 ± 1	34 ± 2	621 ± 74
L-Tryptophan ($N = 14$)	125	17 ± 3†	55 ± 3†	506 ± 83
p-CPA ($N = 12$)	300	5 ± 1†	5 ± 3†	
p-CA ($N = 14$)	10	6 ± 1†	4 ± 2†	
Pargyline ($N = 10$)	10	18 ± 3†	58 ± 6†	

*Data are expressed as means ± S.E.M. †Difference is significant, $F(30, 4) = 5.03$, $P \leq .01$.

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14. B. M. Djuricic and B. B. Mrsulja, *Brain Res.* **138**, 561 (1977). Brains were removed from freshly decapitated rats and, under 3-diopter magnification, were stripped of meninges (containing pia and extracerebral arteries), choroid

plexus, pineal body, and brainstem. Forebrains were homogenized in ten volumes of Earle's solution in Hepes buffer (*p*H 7.4) by a hand-held Potter-Elvehjem homogenizer (0.1-mm clearance; 30 up-and-down strokes). The homogenate was centrifuged at 1500*g* for 10 minutes; the pellet was resuspended in 0.25*M* sucrose and layered over a gradient (1.0 to 1.5*M* sucrose). The gradients were centrifuged at 58,000*g* for 45 minutes at 4°C in a Beckman SW 27 rotor. The microvessel pellet was transferred to a 153-μm nylon mesh, washed with cold saline, and either used directly or frozen and stored in liquid nitrogen until use.

15. The purity of our preparation was judged by the following criteria. (i) The absence of neuronal cells or glial cells, as determined by light microscopic examination of tissue stained with hematoxylin and eosin, phosphotungstic acid-hematoxylin (for glial fibers), elastin Von Gieson stain (for elastin fibers), or toluidine blue (for mast cells). Serial sections of this tissue also did not contain any of those cells or fibers. (ii) The absence of contaminating radioactivity from isotopically labeled synaptosomal or 1500*g* supernatant proteins (35*S*-methionine) when these fractions were added in significant amounts to untreated forebrain tissue just prior to microvessel preparation (I. Gozes and M. Moskowitz, unpublished observation). (iii) The absence of any measureable amount of prostaglandin D_2, the major prostaglandin in brain homogenates [M. Saeed Abdel-Halim, M. Hamberg, B. Sjoquist, E. Anggard, *Prostaglandins* **14**, 633 (1977)] in an incubation mixture containing brain microvessels (M. Moskowitz, unpublished observation). (iv) The presence of the putative transmitter, substance P, in amounts that are less than 0.1 percent of surrounding bovine brain tissue (D. Pettibone, S. E. Leeman, M. A. Moskowitz, unpublished observations).
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18. Rat brain microvessels (2 to 4 mg) were homogenized in 400 μl of 2*M* formic acid:ethanol (1:1), from which a portion was removed for protein determination. Duplicate 150-μl portions of a 35,000*g* supernatant were assayed by a modified version of a previously published assay [see Saavedra *et al.* (*16*)]. The reaction mixture was extracted into 3 ml of chloroform, washed with an equal volume of 1 m*M* HCl and dried with nitrogen gas. The residue was dissolved in 75 μl of absolute ethanol and applied to thin-layer chromatography plates, which were developed unidimensionally to a height of 10 cm, removed, and allowed to dry in a vertical position. This procedure was repeated eight times with pure chloroform as the solvent. Spots corresponding to authentic melatonin were scraped into scintillation vials, eluted with 250 μl of ethanol, and counted for tritium in 3 ml of toluene phosphor.
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Correct Axonal Regeneration After Target Cell Removal in the Central Nervous System of the Leech

Abstract. *The unique target neuron of a severed axon in the leech was selectively eliminated by intracellular injection of protease. In the absence of the target, the severed axon regenerated normally along its original pathway to the usual site of synapse, where it stopped growing without forming alternative connections.*

During development, the nervous system is assembled by the selective growth of its constituent neurons that contact certain target cells while rejecting others. In addition, many neurons in adults can regenerate severed axons to connect with their original targets, thereby restoring function to the nervous system. What role the target plays in axonal growth and in selectivity of connections is unclear. Interactions between growing neurons and their targets in the central nervous system (CNS) have been studied in populations of cells, often after disruptive surgical manipulations. Although conditions of growth during regeneration and development may not be identical, the similarities in the processes are striking.

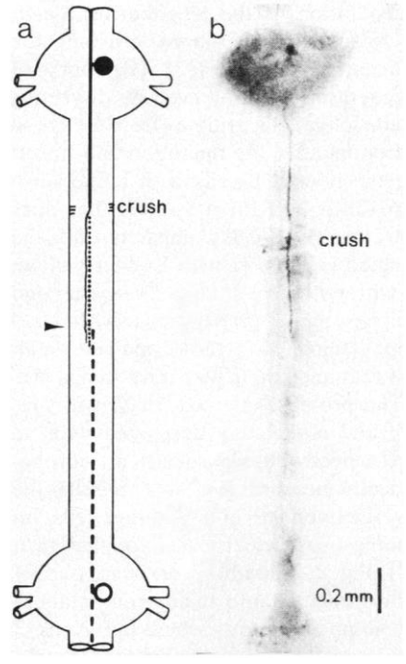

Fig. 1. Regenerating neuron in the ganglion at the top (anterior) in 1 month grows across the crush, along its severed distal stump [dotted line in (a)], and stops at the original synaptic region in the connective midpoint even when the target neuron [dashed line and open circle in (a)] has been selectively destroyed. (b) The regenerating neuron has been marked with intracellular injection of horseradish peroxidase 45 days after its axon was severed (*crush*) and the posterior S-cell (in lower ganglion) killed by an intracellular injection of protease. More extensive growth is not seen later. Arrowhead in (a) indicates the location of cross sections in Fig. 2.

Although the initial directed outgrowth of axons is apparently independent of either the target's location or its presence (*1*), the loss of synaptic targets can lead to the formation of aberrant synapses (*2*). However, recent studies of regenerating motor neurons in the frog have shown that extracellular cues are sufficient to direct the formation of synaptic terminals at the proper sites when the target muscle cells are removed without disturbing the surrounding environment (*3*). It would be useful, therefore, to remove the normal target of a single growing neuron in the CNS without disturbing its environs to determine which aspects of growth, if any, are affected. We now describe the regeneration of an identified axon in the CNS of the medicinal leech when its unique target neuron is selectively eliminated. In the absence of its target, this axon regenerates normally to its usual region of synapse without making aberrant connections.

The system of S-interneurons in the leech allows direct identification of regenerating and target cells. In each segmental ganglion there is one S-cell; it extends an axon both anteriorly and posteriorly halfway along the connectives, the axon bundles that link adjacent ganglia, to make an electrical synapse with the tip of the next S-cell axon (*4*). Fluorescent

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