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Axotomy induces the expression of vasopressin receptors in cranial and spinal motor nuclei in the adult rat

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ABSTRACT **8-L-Arginine vasopressin ([Arg^8]VP) receptors are expressed transiently in the rat facial nucleus during the perinatal period. Electrophysiological studies suggest that at least part of these receptors is located on facial motoneurones. In the present study we report that, in the adult rat, unilateral section of a facial nerve results in a massive and transient reexpression of [Arg^8]VP receptors in the deefferented facial nucleus. Data were obtained by quantitative film autoradiography. During the first 2 postoperative weeks, binding of an iodinated ligand selective for V_{1a}-type receptors increased about 10-fold. Maximal levels of binding were maintained for 1–2 weeks and then started to decrease. Binding was not strictly restricted to the facial nucleus but included the neuropile between motoneuronal pools and the perifacial area, which may indicate a dendritic localization of [Arg^8]VP receptors. To investigate whether other motor nuclei also react to axotomy by up-regulating [Arg^8]VP receptors, we sectioned either a hypoglossal nerve or a sciatic nerve. Two weeks after surgery, the hypoglossal nucleus or sciatic motoneuronal pools ipsilateral to the lesion were intensely labeled with the iodinated ligand. In contrast, nerve section had no effect on oxytocin binding sites in facial, hypoglossal, or sciatic motor nuclei. The results suggest that [Arg^8]VP receptor expression in motor nuclei may depend upon neuromuscular contacts and, thus, that [Arg^8]VP may be involved in the establishment of neuromuscular connections during development and in their restablishment after nerve injury.**

Several lines of evidence suggest that the neuropeptide 8-L-arginine vasopressin ([Arg^8]VP) acts as a neurotransmitter or a neuromodulator in the mammalian brain (1). In particular, [Arg^8]VP can increase the excitability of neurones in regions that contain high-affinity [Arg^8]VP-binding sites in the rat brain, thus suggesting the presence of functional neuronal [Arg^8]VP receptors in these areas (2).

Several regions undetected with [Arg^8]VP receptor ligands in the adult rat brain are transiently labeled during development (3, 4). For instance, the facial nucleus contains numerous V_{1a}-type [Arg^8]VP-binding sites during the few days preceding birth and the first 2 postnatal weeks, a period when [Arg^8]VP can excite a majority of facial motoneurones by a direct postsynaptic effect in brain slices (4, 5). It is conceivable that, in embryos and neonates, endogenous [Arg^8]VP could affect the movements of facial musculature. In addition, [Arg^8]VP could play a role in the maturation of neuromuscular connections. For instance, the multiple innervation of myotubes is lost during the first 2 weeks of life (6), this synaptic rearrangement being affected by the level of activity in motor nerves (7).

The rat facial nucleus and its efferent nerve are extensively used as an experimental system to study how motoneurones respond to separation from their targets by axotomy. In the facial nucleus as in other motor nuclei, motoneurones die if their axons are cut shortly after birth (8), whereas if the section is performed in the adult, they survive, regenerate their axon, and reinnervate their targets (9).

The retrograde changes occurring in motoneurones and their surrounding nonneuronal cells during motor nerve regeneration have been investigated in great detail (9–11). Overall, the consequence of axotomy in the motoneurone is the reappearance of developmental features associated with neurite growth. In contrast, the production of substances involved in synaptic transmission is reduced.

The present study was carried out to assess whether facial nerve section in adult rats would induce the reexpression of [Arg^8]VP receptors in the facial nucleus and, if so, determine its time course. We also studied the effect of axotomy in another cranial motor nucleus, the hypoglossal nucleus, and in spinal motor nuclei of the sciatic nerve. These are regions that also contain more [Arg^8]VP-binding sites in the perinatal period than in adulthood.

MATERIAL AND METHODS

Animals and Surgery. Experiments were performed on adult male rats from a Sprague–Dawley-derived strain. They had free access to water and food pellets. Lights were on from 0700 to 1900, and temperature was maintained around 20°C. Surgery was performed at the age of 4–5 weeks under pentabarbital anesthesia.

Altogether, 39 rats were used. In 35 animals, the right facial nerve was cut about 1 mm distal to the stylomastoid foramen. All branches of the nerve were sectioned, with the exception of the posterior auricular branch. Groups of 5–10 animals were subjected to nerve section on the same day and killed at various postoperative times; their brain was cut in coronal sections and processed in a single autoradiographic experiment. The 6 animals of the first group were killed 3–28 days after nerve section; the 10 rats of the second group survived 1–44 days after surgery; the 10 rats of the third group were killed in pairs on postoperative days 8, 10, 13, 16, and 20, respectively; the 9 animals of the fourth group were killed after 4–35 days. In 2 of the remaining 4 animals, the right hypoglossal nerve was exposed at the level of the digastric muscle and cut. In the other 2 remaining rats, the sciatic nerve was exposed and cut in the thigh. These last 4 animals were killed 14 days after surgery. In all animals, the side controlateral to the nerve section served as control.

Chemical Compounds. To detect the [Arg^8]VP receptors, we used a recently synthesized vasopressin antagonist (VPA), HO-Phaa-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-

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Abbreviations: [Arg^8]VP, 8-L-arginine vasopressin; OT, oxytocin; VPA, vasopressin antagonist; HO-Phaa-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-NH_2, where HO-Phaa = hydroxyphenylacetyl, D-Tyr(Me) = *O*-methyl-D-tyrosine, and Arg-NH_2 = argininamide; OTA, the OT antagonist [β-mercapto-β,β-pentamethylenepropionyl^1,Tyr(Me)^2,Thr^4,Orn^8,Tyr-NH_2^9]OT, where Orn = ornithine.

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NH_2, where HO-Phaa = hydroxyphenylacetyl, D-Tyr(Me) = *O*-methyl-D-tyrosine, and Arg-NH_2 = argininamide (see ref. 12). This compound was monoiodinated on the phenolic residue in position 1 to yield ^{125}I-labeled VPA (^{125}I-VPA), a ligand highly selective for V_{1a} receptors (13). The specific oxytocin (OT) antagonist (OTA), [β-mercapto-β,β-pentamethylenepropionyl^1,Tyr(Me)^2,Thr^4,Orn^8,Tyr-NH_2^9]OT (where Orn = ornithine; ref. 14), was iodinated in position 9 (15). Radioiodinations yielded specific activities of about 2000 Ci/mmol. VPA and OTA were provided by M. M. Manning (Medical College of Ohio, Toledo). [Arg^8]VP and OT were purchased from Bachem or from Novabiochem.

Tissue Preparation and Autoradiography. Animals were killed under pentabarbital anesthesia; the brainstem and the lumbosacral part of the spinal cord were removed and frozen in 2-methylbutane at −25°C. Serial coronal sections (14 μm thick) were cut, laid on gelatin/chrome alum-coated slides, and stored at −80°C until use. To label [Arg^8]VP receptors, we used ^{125}I-VPA. Sections were lightly fixed by dipping the slides for 5 min in a solution of 0.2% paraformaldehyde in phosphate-buffered saline (pH 7.4) and then rinsed for 15 min in 50 mM Tris·HCl buffer (pH 7.4) supplemented with 0.1% bovine serum albumin. Each slide was thereafter covered with 400 μl of incubation medium (50 mM Tris·HCl/0.025% bacitracin/5 mM $MgCl_2$/0.1% bovine serum albumin) containing 0.03–0.05 nM ^{125}I-VPA. Nonspecific binding was determined by incubating adjacent sections in medium containing, in addition to ^{125}I-VPA, 1 μM nonradioactive [Arg^8]VP. Incubation was carried out at room temperature for 1 hr in a humid chamber under gentle agitation. It was followed by two 5-min washes in ice-cold incubation medium and a quick rinse in distilled water. Slides were then dried in a stream of cold air and placed in an x-ray cassette in contact with β_{max} Hyperfilm (Amersham) for 3–6 days. Films were developed in Kodak D19 for 5 min, and the sections were stained with cresyl violet. OT receptors were labeled in adjacent sections by the same procedure except that the incubation medium contained 0.07 nM of the radioiodinated OTA. Nonspecific binding was assessed by using the same amount of ligand together with 1 μM of nonradioactive OT on adjacent sections.

The specific binding of ^{125}I-VPA was quantified in all animals with facial nerve section by using a charge-coupled device (CCD) videocamera with 255 levels of grey and the SAMBA system analysis for personal computers (Alcatel, Grenoble, France). For each section analyzed, the surface occupied by the facial nucleus and the perifacial area was measured on the cresyl violet-stained section, and the mean OD of the film in this surface was determined. For each animal, every eighth section was analyzed throughout the whole extension of the right facial nucleus (10–15 sections analyzed per nucleus). OD values were converted into fmol/mg of tissue from standard curves derived from coexposed standards. Results are expressed as total specific binding in femtomoles of ^{125}I-VPA per facial nucleus. This was estimated to equal the product of the facial nucleus volume (mm^3) × mean ^{125}I-VPA binding (fmol/mg of tissue). We assume brain density to be 1 mg/mm^3.

RESULTS

In normal adults rats, the facial nucleus shows low levels of [Arg^8]VP-binding sites in its medial part. Unilateral facial nerve section induces a massive and sustained expression of [Arg^8]VP-binding sites in the ipsilateral facial nucleus. In an autoradiogram obtained from an animal in which the right facial nerve had been cut 20 days prior to death, intense labeling was observed throughout the right facial nucleus, while the left nucleus appeared to be almost unlabeled (Fig. 1*A*). This labeling was specific because it was prevented by low concentrations of nonradioactive [Arg^8]VP, as was the labeling in the nucleus of the solitary tract and the choroid plexuses.

Comparison of film autoradiograms with cresyl violet-stained sections showed that the increase of ^{125}I-VPA binding induced by facial nerve section was highest in the intermediate and dorsolateral subdivisions of the facial nucleus (compare Fig. 1 *B* and *C*). The integrity of the posterior auricular branch in our experiments on adult rats probably explains the low density of binding in the ventromedial subdivision. Interestingly, however, the pattern of labeling in the facial nucleus of neonatal unoperated pups also shows more [Arg^8]VP-binding sites in the intermediate subdivision than in other parts of the nucleus.

Labeling observed with ^{125}I-VPA after facial nerve section was not restricted to areas containing motoneuronal cell

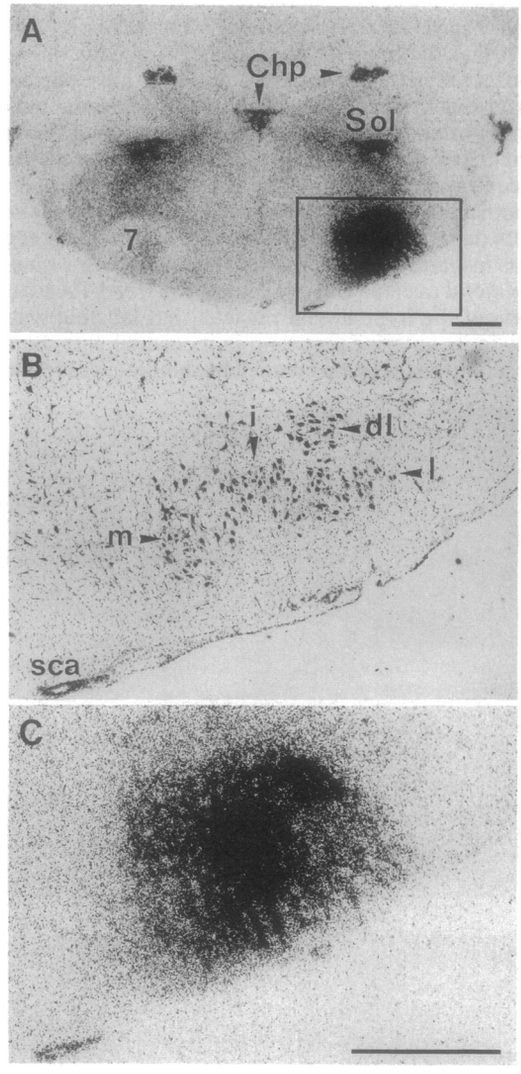

FIG. 1. [Arg^8]VP sites in the brainstem of an adult rat following facial nerve section. (*A*) Autoradiogram obtained from a rat killed 20 days after the section of the right facial nerve showing a dense specific ^{125}I-VPA binding in the facial nucleus (7) on the operated side (boxed). In contrast, the intact left facial nucleus is almost unlabeled. Other labeled areas are the nucleus of the solitary tract (Sol), the choroid plexuses (Chp). (*B*) Rectangular zone outlined in *A* on the section used to generate the autoradiogram, stained with cresyl violet. m, medial; i, intermediate; dl, dorsolateral; l, lateral; sca, superior cerebellar artery. (*C*) Same area as *B* on the autoradiogram. Note that axotomy-induced ^{125}I-VPA binding extends in the perinuclear neuropile, in particular in the area ventral to the nucleus where it forms bundles. Note also that the labeling is densest in the intermediate and dorsolateral parts of the nucleus. (Bars = 1 mm.)

bodies but extended over the surrounding neuropile. This was particularly evident in the caudal half of the nucleus, where labeling occurred as bundles crossing the zone between the ventral edge of the nucleus and the ventral surface of the brain (Fig. 1*C*). A similar arrangement in bundles of dendrites immunoreactive for choline acetyltransferase is seen in this zone in normal animals (unpublished data). Thus, axotomy-induced [Arg^8]VP-binding sites in the facial nucleus area may be localized on dendrites.

The effect of axotomy on the expression of [Arg^8]VP-binding sites in the facial nucleus was examined at various times after nerve section. Fig. 2 shows autoradiograms obtained from one group of animals. Quantitative analysis of the autoradiograms from this experiment shows that ^{125}I-VPA binding increased ≈10-fold (Fig. 3*A*) during the 3 postoperative weeks. Thereafter, the amount of ^{125}I-VPA binding began to decline slowly. Furthermore, the data suggest that the increase is biphasic. A similar time course was observed in the other experimental groups, one of which is illustrated in Fig. 3*B*. The number of ligand molecules bound reported in Fig. 3 represents roughly half the number of facial [Arg^8]VP-binding sites, since ^{125}I-VPA was used at a concentration close to its dissociation constant.

In both animals subjected to hypoglossal nerve section and killed 14 days thereafter, ^{125}I-VPA labeling of the hypoglossal nucleus ipsilateral to the lesion was much denser than in the controlateral nucleus (Fig. 4). Binding of ^{125}I-VPA was found throughout the hypoglossal nucleus, but labeling was more intense in its anterior part than in its caudal part. Similar to the observation in the facial nucleus, binding was found to be associated not only with groups of motoneuronal cell bodies but also was extended into the surrounding neuropile.

In the lumbosacral spinal cord of normal adult rats, the gray matter is moderately and evenly labeled with ^{125}I-VPA. Fourteen days after a unilateral section of the sciatic nerve, the density of specific binding was markedly increased in lateral motor nuclei ipsilateral to the lesion (Fig. 5). Enhanced labeling was observed from the caudal part of L3 segment to the rostral part of S1 segment but was most intense in L4 and L5.

In adult rats, neither facial, nor hypoglossal, nor sciatic nerve section affected binding of the iodinated OTA in the corresponding motor nuclei or in nearby regions.

DISCUSSION

The main finding of the present study is that section of a facial nerve in the adult rat leads to a massive and slowly reversible reexpression of [Arg^8]VP-binding sites within its facial nucleus. Peripheral axotomy also increases the number of [Arg^8]VP-binding sites in the hypoglossal nucleus and in sciatic motor nuclei.

Axotomy-induced [Arg^8]VP-binding sites were detected with a new linear antagonist selective for V_{1a}-type [Arg^8]VP receptors (12, 13). With this ligand, we found the same developmental pattern of [Arg^8]VP-binding sites in the facial nucleus as with [^{3}H][Arg^8]VP—i.e., high amounts of binding until postnatal day 10 and almost undetectable levels after 3–4 weeks of age (4). In the hypoglossal nucleus, the density of [Arg^8]VP-binding sites present in young animals is much

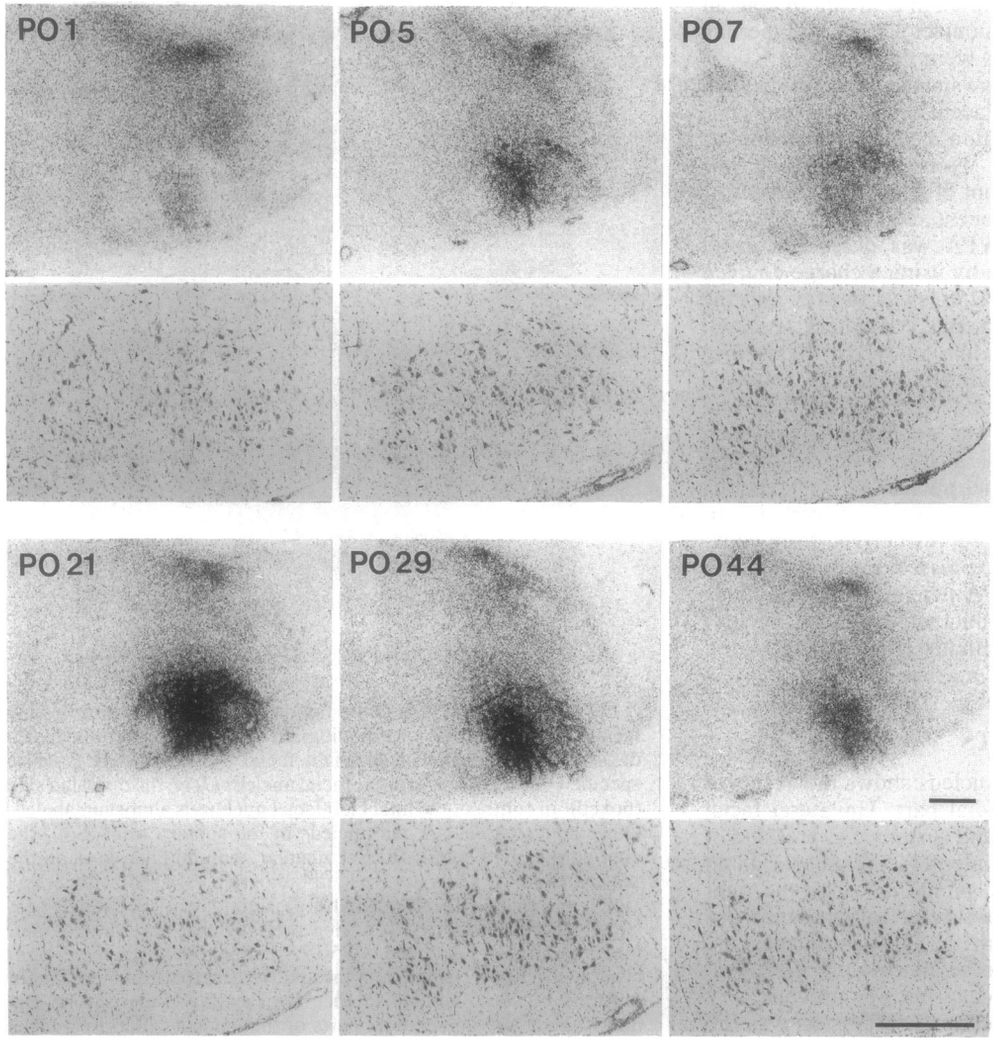

FIG. 2. [Arg^8]VP-binding sites in the facial nucleus at various postoperative (PO) days after axotomy. Data are from six animals of group 2, which is also illustrated in Fig. 3*A*. (*Upper*) Autoradiograms. (*Lower*) Photographs of the facial nucleus area on the cresyl violet-stained sections used to generate the corresponding autoradiograms, showing that they originate from approximately the same anteroposterior level of the nucleus. Note the marked difference in density and extent of ^{125}I-VPA binding at various postoperative days. (Bars = 500 μm.)

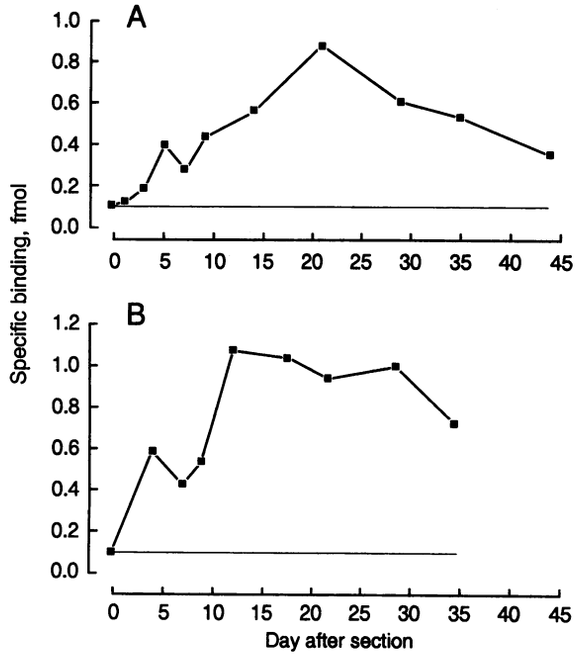

FIG. 3. Time course of the effect of axotomy on the number of [Arg^8]VP-binding sites in the facial nucleus. Data are from two groups of animals. (*A*) Group 2. (*B*) Group 4. Each point represents a single animal and shows the total amount of specific ^{125}I-VPA binding in the facial nucleus ipsilateral to the lesion (ordinate) as a function of postoperative time (abscissa). The horizontal line represents the mean level of specific binding in the controlateral control facial nucleus.

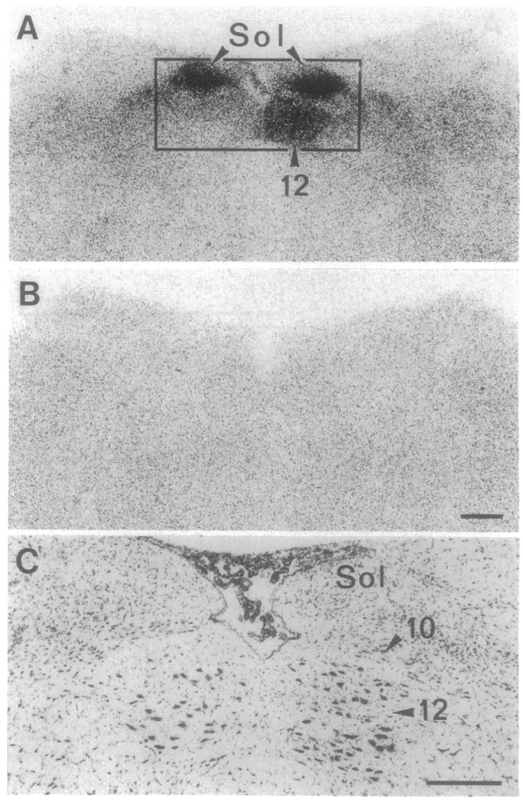

FIG. 4. [Arg^8]VP-binding sites in the brainstem 14 days after section of the right hypoglossal nerve. (*A* and *B*) Autoradiograms from adjacent sections incubated with ^{125}I-VPA either alone (*A*) or in the presence of an excess of nonradioactive [Arg^8]VP (*B*). (*C*) Area outlined in *A* of the cresyl violet-stained section used to produce autoradiogram *A*. Note the intense labeling of the right hypoglossal nucleus (12) ipsilateral to the nerve section. Positioned dorsally to the hypoglossal nucleus, the nucleus of the solitary tract (Sol) is labeled on both sides, while the dorsal motor nucleus of the vagus nerve (10) is unlabeled. (Bars = 500 μm.)

lower than in the facial nucleus but is similarly reduced in adults (4, 16). In the lumbar spinal cord, [Arg^8]VP-binding sites are evenly distributed within the gray matter both in young and adult rats, although in higher amounts in young animals (unpublished data).

The amount of OT-binding sites in the corresponding motor nuclei or in nearby regions where such sites are detectable transiently during development (17) was not affected by either facial, hypoglossal, or sciatic nerve section. Thus, we postulate that the increased production of [Arg^8]VP receptors in the facial, the hypoglossal, and the sciatic motor nuclei after axotomy has some specificity and indicates a function for [Arg^8]VP in the processes of motor nerve regeneration and muscle reinnervation.

Our autoradiographic data suggest that the increase of facial [Arg^8]VP-binding sites caused by nerve section is biphasic. Interestingly, the synthesis of calcitonin gene-related peptide, a signaling factor probably involved in both neuroglial (18) and neuromuscular (19) interactions during motor nerve regeneration, is increased in regenerating facial motoneurones with a similar time course (20, 21).

Most of the morphological and chemical changes seen in axotomized motoneurones suggest that the injured cells undergo a process of dedifferentiation and alter their metabolism towards a "growing" rather than a "transmitting" mode (9–11). Thus, the synthesis of cytoskeletal proteins and growth-associated proteins is increased, whereas transmitter-associated enzymes and receptor proteins for neurotransmitters are decreased as long as contact with targets is not reestablished. Along the same line, binding of ligands to receptors for neurotransmitters is reduced as well as the number of afferent presynaptic terminals.

In this context, the up-regulation of [Arg^8]VP receptors in motor nuclei following axotomy may indicate a function for [Arg^8]VP in neuronal recovery and regeneration that is distinct from its potential role in synaptic transmission. Thus, it is noteworthy that the expression of the receptor for transferrin, a substance known to have a critical role in several growth processes (22), is markedly up-regulated in axotomized motoneurones (23). Similarly, the expression of nerve growth factor receptor mRNA is developmentally regulated and increased in rat spinal cord motoneurones after axotomy (24). However, the physiological function of nerve growth factor on motoneurones remains unclear at present. It was also reported recently that ciliary neurotrophic factor receptor mRNA is increased in spinal cord of adult rats after sciatic nerve section (25). Thus, a compound known to prevent motoneuronal death after axotomy at birth (26) may also be involved in the response of adult motoneurones to nerve lesion.

While it is recognized that [Arg^8]VP is a mitogenic factor in a variety of nonneuronal cells, the possibility that [Arg^8]VP could act as a neurotrophic factor has not yet been investigated extensively. However, [Arg^8]VP has been shown to promote neurite outgrowth of cultured embryonic nerve cells from *Xenopus laevis* (27) and to potentiate the effects of 3,3',5-triiodothyronine on survival and maturation of cholinergic rat hippocampal neurones (28). On the other hand, the [Arg^8]VP-deficient, homozygous Brattleboro rat exhibits impaired brain development. However, the most severely affected parameter is neurogenesis in the cerebellum (29), an area that has not been shown to contain [Arg^8]VP receptors (3).

In parallel with the events initiated by axotomy in motoneurones, changes take place in the surrounding glial cells,

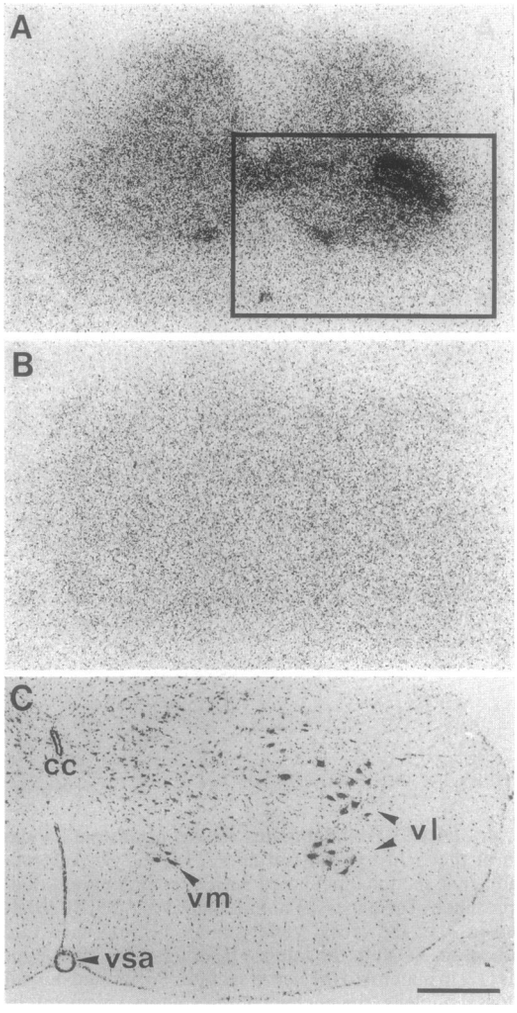

FIG. 5. [Arg^8]VP-binding sites in the L5 segment of the spinal cord 14 days after section of the right sciatic nerve. (*A*) Autoradiogram of a coronal spinal cord section incubated with ^{125}I-VPA. (*B*) Autoradiogram from a section adjacent to *A* incubated with ^{125}I-VPA in the presence of nonradioactive [Arg^8]VP in excess. (*C*) Area outlined in *A* of the cresyl violet-stained section used to generate autoradiogram *A*. On the operated side, dense specific binding is present in the motoneurone-rich ventrolateral gray matter (vl). Specific binding is also present bilaterally in a ventromedial group of motoneurones (vm) and in the ventral spinal artery (vsa). cc, central canal. (Bar = 500 μm.)

the most striking being the proliferation of microglial cells and the morphological transformation of astrocytes (for a recent review, see ref. 11). Our study does not allow us to decide which facial or hypoglossal cells bear [Arg^8]VP receptors after nerve section. A microglial localization of [Arg^8]VP receptors is unlikely because the microglial reaction to axotomy lasts only ≈2 weeks (30). A neuronal localization is suggested by electrophysiological recordings from brain slices of neonate rats, which have shown that virtually all facial (5) and hypoglossal motoneurones (16) respond to exogenous [Arg^8]VP. However, the corresponding cells do not survive in slices of adult rat brain. Therefore, the possibility that [Arg^8]VP receptors expressed in motor nuclei during development or after axotomy are also present on reactive astrocytes cannot be excluded.

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